RECOMBINANT *BACILLUS SUBTILIS* SPORES AS A SAFE CARRIER FOR ENTERIC IMMUNIZATION AGAINST *ECHINOCOCCUS GRANULOSUS*

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Abstract

The *Echinococcus granulosus* tapeworm infects intermediate hosts as humans and sheep, inducing Echinococcosis that leads predominantly to liver cysts. An effective manner for preventing *E. granulosus* infection of the intermediate hosts, is through the interruption of its life cycle, by directly treating the definitive hosts, as are dogs.

Bacillus subtilis is an extensively studied Gram-positive bacteria that possess several advantages, as i) the generation of robust spores; which resist to harsh environmental conditions; ii) it is genetically tractable; iii) considered as a safe probiotic, making of this bacteria a good candidate for an enteric vaccine approach. Additionally, *B. subtilis* develops in biofilms, which are bacterial communities with an extracellular matrix. TasA, a main biofilm matrix protein, when fused at the C-terminal with the fluorescent protein mCherry showed to be distributed in the whole biofilm. By taking advantage of the widespread distribution of TasA in the biofilm, we intended to express *E. granulosus* antigens, as tropomyosin (EgTrp) and paramyosin (EgA31) fused at the C-terminal of TasA. Thus, spores of recombinant *B. subtilis* strains for these antigens had been orally provided to animals with the aim of bypassing the stomach barrier, germinate in the gut and develop in a biofilm that will expose on its surface the antigens of interest. These antigens would eventually stimulate the gut-associated lymphoid tissues allowing the establishment of a humoral and cellular immune response against the *E. granulosus* tapeworm. To test this hypothesis, series of *B. subtilis* were engineered for carrying antigenic peptides for EgTrp and EgA31. The obtained recombinant and wild-type *B. subtilis* spores showed the same resistance to harsh conditions as are high temperature, shelf-life survival, and acidic conditions. In a first instance, when testing the recombinant spores in orally administered Balb/c mice, the spores showed to be able to germinate after the passage through the gastrointestinal tract. Interestingly, mice developed a significantly increased humoral immune response, detectable as secreted IgA in the feces when inoculated with spores carrying (102-278)EgTrp. In a second instance, when treating mice with an antibiotic cocktail (to favor the *B. subtilis* germination in the gut) previous to the spore oral gavage, the mice developed a significant increase in local and systemic humoral immune response to both EgTrp antigenic peptides, (102-207)EgTrp and (102-278)EgTrp. However, the mice receiving recombinant spores carrying EgA31 antigenic peptides were unable to develop a detectable humoral or cellular immune response under any of the tested conditions.

The recombinant spores were also tested in dogs, as they are the main target group for a potential vaccine against echinococcosis. The results obtained from the spore

administration in dogs were similar to the mouse experimentation. Thus, one out of two dogs receiving recombinant spores for EgTrp and one out of two dogs receiving the mixture of spores (EgTrp and EgA31) can elicit an immune response. However, dogs receiving EgA31 showed signs to develop a tolerance towards paramyosin, as one dog was showing an increase in proliferation of T-regulatory cells during the experiment. This data was consistent with the result obtained from mouse experimentation, indicating that paramyosin is not a good antigen for enhancing the humoral immune response.

Finally, we were able to develop a carrier. The *B. subtilis* spores based carrier of recombinant *E. granulosus* antigens resulted in a significant increase of detected antibodies in mice and an increase of antibodies in dogs against components of the recombinant *B. subtilis* biofilm of the (102-207)EgTrp strain. However, this potential vaccine needs to be challenged in dogs with *E. granulosus* tapeworms to prove its real efficacy.

Zusammenfassung

Der Hundebandwurm *Echinococcus granulosus* infiziert Zwischenwirte (z.B. Menschen und Schafe) und verursacht Echinococcosis. Die Infektion mit *E. granulosus* führt vornämlich in der Leber zu Bildung von Zysten. Ein effektiver Weg um Zwischenwirte vor einer solchen Infektion zu schützen, ist den Hauptwirt (z.B. Hunde und Wölfe) gegen den Bandwurm zu behandeln.

Bacillus subtilis ist ein gut erforschtes Gram-positives Bakterium mit einigen Vorzügen, wie zum Beispiel: i) die Bildung von robusten Sporen, welche harschen Umweltbedingungen widerstehen, ii) die genetische Modifizierbarkeit und iii) die Anerkennung als sicheres Probiotikum, machen dieses Bakterium zu einem guten Kandidaten für einen enteralen Impfstoff. Zusätzlich bilden *B. subtilis* Bakterien Verbünde, geschützt durch eine extrazelluläre Matrix, sogenannte Biofilme. TasA, ein Hauptprotein der extrazellulären Matrix, zeigt sich über die gesamte Oberfläche des Biofilms verteilt, wenn man es C-terminal mit einem fluoreszierenden mCherry Protein fusioniert. Den Vorteil nützend das TasA so verteilt ist im Biofilm, beabsichtigten wir die *E. granulosus* Antigene Tropomyosin (EgTrp) und Paramyosin (EgA31) an den C-Terminus von TasA zu fusionieren.

Sporen von rekombinanten *B. subtilis* Stämmen mit diesen Antigenen werden oral an Tiere verabreicht. Dies mit dem Ziel, dass die Sporen den Magen passieren, im Darm germinieren, und sich zu einem Biofilm entwickeln. Dadurch würden die Antigene exprimiert. Diese Antigene werden möglicherweise die Darm-assoziierten lymphatischen Gewebe stimulieren und zu einer humoralen und zellulären Immunantwort gegen *E. granulosus* führen. Zu diesem Zweck wurden Stämme von *B. subtilis* entwickelt welche die Peptide EgTrp und EgA31 exprimieren können. Die erhaltenen rekombinanten sowie auch die wildtyp *B. subtilis* Sporen zeigten eine starke Widerstandsfähigkeit gegen äussere Einflüsse wie hohe Temperaturen, Langzeit-Exposition zu Raumtemperatur und sauren Bedingungen.

In einem ersten Schritt zeigten Sporen die Fähigkeit, dass sie die Passage durch den Gastrointestinal-Trakt überstehen. Interessanterweise entwickelten Mäuse ein signifikanter Anstieg der humoralen Immunantwort welche sich durch sekretierte IgA im Stuhl von mit (102-278)EgTrp inokulierten Mäusen zeigte. In einem zweiten Schritt wurden die Mäuse vor der Gabe von rekombinanten Sporen mit Antibiotika behandelt um die germinierung von *B. subtilis* zu begünstigen. In diesem Fall entwickelten die Mäuse eine lokale und systemische humorale Immunantwort gegen beide EgTrp Varianten, (102-207)EgTrp und (102-278)EgTrp. Im Gegensatz dazu, entwickelten Mäuse welche

EgA31 exprimierende rekombinante *B. subtilis* verabreicht erhielten keine humorale oder zelluläre Immunantwort unter den getesteten Bedingungen.

Die rekombinanten Sporen wurden auch an Hunden getestet, da diese die Zielgruppe für einen Impfstoff gegen Echinococcosis darstellen. Die Resultate waren vergleichbar zu den Resultaten aus den Maus Experimenten. Einer von zwei Hunden die EgTrp Sporen erhielten und einer von zwei Hunden die ein Gemisch aus EgTrp und EgA31 Sporen erhielten, entwickelten eine Immunantwort gegen Komponenten des Biofilms des (102-207)EgTrp *B. subtilis* Stamms. Im Gegensatz dazu entwickelte ein Hund welcher rekombinante EgA31 Sporen erhielt Zeichen einer Toleranz gegen Paramyosin.

Abschliessend können wir festhalten, dass wir einen Träger basierend auf *B. subtilis* Sporen entwickelten und dieser zu einem vorsichtig positiven Resultat in der oralen Immunisierung von Mäusen und Hunden gegen *E. granulosus* führte. Jedoch muss der Impfstoff mit einer Behandlung der Hunde mit *E. granulosus* Bandwürmern getestet werden, um die wirkliche Effizienz zu beweisen.

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List of Abbreviations

Ab	Antibody
APC	Allophycocyanin
B. subtilis	*Bacillus subtilis*
B. subtilis wt	*B. subtilis* NCIB3610 wild type
BSA	bovine serum albumin
C-terminal	carboxy-terminal
CE	cystic Echinococcosis
CFU	colony forming units
CFSE	Carboxyfluorescein succinimidyl ester
Cm	Chloramphenicol
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DPA	pyridine-2,6-dicarboxylic acid (dipicolinic acid)
DSM	Dyfco sporulation media
dy	daily
E. coli	*Escheria coli*
E. granulosus	*Echinococcus granulosus*
ECM	Extracellular matrix
EgA31	*E. granulosus* Paramyosin
EgTrp	*E. granulosus* Tropomyosin
ELISA	Enzyme linked immunosorbent assay
EPS	Exopolysaccharide
Ex	Excitation
FC	Flow cytometry
FITC	Fluorescein
GALT	Gut associated lymphoid tissue
GAVI	Global Alliance for Vaccines and Immunization
GIT	Gastro intestinal tract
GRAS	Generally regarded as safe
H	Hour
IF	Immunofluorescence
IL	Interleukin
IPTG	Isopropyl-β -D-thiogalactopyranoside

IPV	Inactivated polio vaccine
HRP	Horse radish peroxidase
kDa	Kilo Dalton
km	Kanamycin
LB	Luria Bertani medium
LL	Laminated layer
M	Microfold cells
mAb	Monoclonal antibody
min	Minute
MLN	Mesenteric lymph nodes
MLS	Erythromycin
NK	Natural killer cells
o.g.	Orogastrically
OD	Optical density
ON	Overnight
OPV	Oral polio vaccine
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE/CY7	Phycoerythrin-cyanine 7
PI	Pre-immune
s	Second
sIgA	Secreted Immunoglobulin A
Spc	Spectinomycin
Th1	T helper cells type 1
Th2	T helper cells type 2
T_{reg}	T regulatory cells
US	The United States of America
V	Volt
WB	Immunoblotting
WHO	World Health Organization
wy	Weekly

Introduction

The history of vaccines

The history of vaccine development is linked to the deadly poxvirus "variola", which causes smallpox in humans for which records go back more than 3500 years [1]. The beginning of the vaccination dates from the first observations reported by Thucydides in 430 BC. He observed that people who survived a severe and contagious disease would not suffer from the same illness a second time [2]. Then the first active immunization was reported during the Middle Age in China, where the so-called “variolation” was practiced. In this procedure, healthy people were exposed to air-dried pustules of smallpox from humans. This procedure was also practiced in Turkey from where the wife of the British ambassador in Istanbul, Lady Mary Wortley Montagu imported the variolation to Britain. However, the variolation procedure was frequently accompanied by severe side effects and often lead to death [3]. In the eighteenth centuries, Edward Jenner, an English physician, and scientist (1749–1823) combined the procedure of variolation with his observation that the milkmaids in contact with the cowpox pustules did not suffer from smallpox. The proof of principle for the vaccination was given by the Jenner's vaccination experiment in 1796. James Phipps, an eight-year-old boy, was vaccinated with a cowpox lesion from a milkmaid. Followed by a smallpox challenge, which remained without adverse effects for James [3,4]. From there on, variolation was performed using cowpox pustules to immunize and protect people against deadly smallpox.

Then, in 1879, Louis Pasteur (1822-1895) did the following important step in the vaccines history, when observing that chickens were not dying of a fowl cholera challenge after a former injection of accidently attenuated cholera bacteria. Pasteur realized that he demonstrated the immunization by artificially attenuated agents. He used this discovery to develop an attenuated anthrax vaccine, which he tested successfully by 1881 [5]. Based on these findings, life attenuated pathogens, and parts of it were used to raise immunity in humans and animals against various diseases.

Apart from protecting the individual animal and its flock from a disease, the animal vaccines have an additional goal to protect humans from anthropozoonoses [6]. Furthermore, the development of vaccines for animals differs from vaccination for people by ethical issues surrounding the experimentation and testing as well as the economic considerations, which are guiding the way of handling a disease. Also, alternative measures can be taken to hinder a disease from spreading, as is, for example, the slaughter of sick animals [6,7]. The vaccination of animals goes back to the inoculation of

sheep with serous fluid from infected animals to protect them from sheep-pox, named as "clavélisation". However, due to the historic vaccination advents, it remains unclear if this kind of inoculation leads to the idea of human variolation or vice versa [6].

The story of animal vaccines continued with Louis Pasteur's finding in 1882 that pathogens affecting one species and passed through another species do not affect the same severity the first species anymore. Which was based on the empirical observation of farmers and veterinarians and he then developed the method of "lapinised vaccines". In this case, Pasteur attenuated the *Erysipelothrix rhusiopathiae* Gram-positive bacteria, the causing agent for swine erysipelas, by injecting it in series to rabbits before inoculating the swine [8]. The discoveries of Jenner and Pasteur had allowed the production of attenuated pathogens for different vaccines used against various pathogens, as diphtheria toxoid (1923), Polio (1963), Adenovirus (1980), Rotavirus (RotaTeq® 2006, Rotarix® 2008), and 21-Valent pneumococcus (2012) [9].

Also, with the progress in science and technology, new modes of vaccination have been introduced, like is the gene-based delivery of antigens, which derive an immune response through synthesizing antigenic proteins within antigen-presenting cells. This methodology has proven its effectiveness, in particular against human influenza virus by using DNA-expression vectors or replication-defective virus [10,11]. Also, other alternative methods of vaccination had been developed as antigenic protein expression through replicons, virus-like particles, live-attenuated viruses, purified antigenic peptides or by recombinant bacteria expressing antigens [9,12–15].

Up to date, 84 different vaccines are available and are licensed in Switzerland for its use in humans (Swissmedic, 31st October 2015 [16]) and 137 vaccines for veterinary purposes. Among those, 23 vaccines are destined for dogs in Switzerland (Institute of Virology and Immunology, 31st October 2015 [17]). However, the current world health conditions are differing from the Swiss sanitary conditions as has been reflected by the estimation of the Global Alliance for Vaccines and Immunization (GAVI) that more than 1.5 million children die annually from vaccine-preventable diseases. Mainly due to the lack of vaccine availability in developing countries because of the economic and political context [9,18]. Besides, there is a need for improvement of vaccines against several important diseases. Few examples are viruses like Dengue, Ebola or HIV and as bacteria *Mycobacterium tuberculosis*, *Mycobacterium lepromatosis* and as parasites *Plasmodium malariae* or the tapeworm *Echinococcus granulosus* [19–21].

Bacillus subtilis: biofilm and spores

A potential vaccine for developing countries needs to be easily administered, independent of a cold chain, cost efficient in production and in a safe biological background [22]. A good candidate corresponds to the spores of *Bacillus subtilis* (*B. subtilis*) because of the possibility to be orally administered, the independence of a cold chain, the low-cost preparation, and purification, the use of safe food supplement and the resistance to harmful chemicals [23–28]. *B. subtilis* is a Gram-positive bacterium commonly present in soil and water [29]. Importantly, *Bacillus* species can establish an endosymbiotic relationship with their host, surviving and proliferating within the gastrointestinal tract (GIT) [30]. For example, it has been described that the spores *B. subtilis var. natto* can germinate within the GIT of mice [31]. *B. subtilis* possess the status of "generally recognized as safe" (GRAS) by the American food and drug administration (FDA GRAS notice 562 [23]). In the GRAS notice are listed ten studies using *B. subtilis* for human ingestion that declare *B. subtilis* as having no adverse effects.

Biofilms: bacterial communities with extracellular matrix

Surface-associated Multicellular communities encased in a self-produced extracellular matrix (ECM) are referred to as biofilms [32–34]. The ECM of biofilms is composed of extracellular polymeric substances, as carbohydrate-binding proteins [35,36], pili, flagella, adhesive fibers [37] and extracellular DNA [38]. The matrix does not only stabilize the biofilm but also traps nutrients and water through H-bond interactions [39]. These structural components of the matrix keep bacteria in close contact and enable direct cell-to-cell interactions as used for DNA exchange [40] and also, protecting the bacteria from dehydration, predation, oxidizing molecules and other damaging agents as antibiotics [40–43]. Biofilms can be problematic in clinical settings as they are harder to treat due to their inherent resistance to environmental insults [41,42,44]. An example of this is the formation of a *Pseudomonas aeruginosa* biofilm in the upper respiratory tract that leads to persistent and recalcitrant infections[45,46] or the urinary tract infections that are induced by the biofilm of the uropathogenic *Escheria coli* [47]. In contrast, there are cutting edge applications in which biofilms can be used to facilitate industrial processes as the bio-catalysis. Some examples of bio-catalysis supported by biofilms correspond to the catalysis of acetic acid, ethanol or butane-2,3-diol [48] and the bio-removal in waste water treatment of heavy metal as is the trivalent chromium using *B. subtilis* biofilms [49].

Bacillus subtilis biofilm formation

B. subtilis strains have the ability to form biofilms [50]. The ECM is formed by proteins, exopolysaccharides, and nucleic acids and corresponds to more than 90% of the dry weight of the biofilms [40]. The ECM proteins are essential in the biofilm structure and can form fibrous amyloid-like structures [35,51,52]. These particular fibers are composed by TasA in the extracellular space and get anchored to the cell wall by TapA [53]. These two proteins, TasA and TapA, are processed and secreted to the extracellular space by the signal peptidase sipW. These three proteins are encoded in the three-gene operon *tapA-sipW-tasA* (*tapA* operon) as schematically represented in **Figure 1** [34,35].

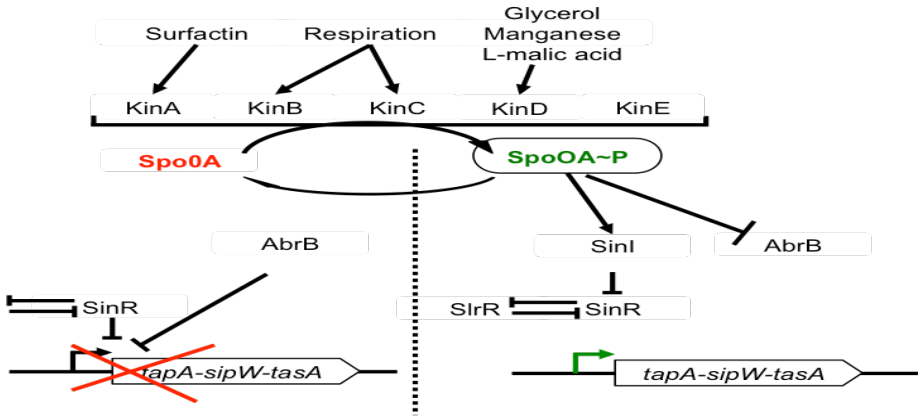


Figure 1. Regulation of the biofilm formation in *B. subtilis* through the Spo0A phosphorylation relay adapted from Mielich-Süss et al. The kinases KinA–E get activated through the indicated pathways, which are triggered by different stimuli as increase of surfactin concentration, respiration or Glycerol, Manganese and L-malic acid. In the absence of spores or matrix forming environmental conditions, Spo0A gets dephosphorylated, and hence, it is inactive. This condition permits the binding of SinR and AbrB to the Tap promoter driving its inactivation. SinR is regulated by SlrR in a double negative feedback loop. By changing environmental conditions the KinA-E get activated and phosphorylate Spo0A. Spo0A~P is inducing the expression of SinI a SinR inhibitor and as well inhibiting AbrB. By this, the tasA operon remains unrepressed any longer and the matrix-related genes are expressed. Adapted from Mielich-Suess et al [60].

Furthermore, the extracellular matrix production of *B. subtilis* is under the control of the ratio of Spo0A to phosphorylated Spo0A~P [54]. A low level of phosphorylated Spo0A~P induces matrix production, and a higher level favors the sporulation of the bacteria whereas the dephosphorylated Spo0A induce a motile form of the bacteria [55]. The phosphorylation and therefore activation of Spo0A is induced by five different kinases (KinA-E) [56]. Spo0A~P is inducing the expression of SinI, which is inhibiting the transcription repressor SinR. The repressor SinR directly binds and inhibits the promoter of the operons *epsA-O* and *tapA-sipW-tasA* coding for the exopolysaccharide (EPS) and

the matrix protein TasA, respectively [35,57]. Also, SinR could be inactivated by SlrA [58,59], which in turns is under the control of the transcription repressor YwcC, then be involved in a double negative feedback loop [60]. However, the signal cascade triggering this second pathway is unknown [61].

The analysis of the activated *tapA* promoter (formerly known as *yqxM*) within a formed biofilm was performed by Vlamakis et al. in 2008 by cloning the *tapA* promoter in front of the fluorescent protein YFP tag into the *lacA* locus. By visualizing the YFP by flow cytometer and fluorescent imaging, they determined the time point and localization in the biofilm of the activation of the *tapA* promoter. They could show that *in vitro* a *B. subtilis* biofilm contains, at 24 h post-inoculation, about 81 % of cells with an active tapA promoter, which are therefore seen as matrix-producing cells. The fraction of matrix-producing (P_{tapA}) cells drops then to 65 % (48 h) and 38 % (72 h) whereas the fraction of cells with activated sporulation (P_{sspB}) promoter increase in numbers. The fraction of motile cells is dropping earliest; 70 % at 12 h and only 26 % at 24 h express the motility promoter P_{hag}. Further Vlamakis et al. emphasize that the sporulation of the bacteria is dependent on the formation of an architecturally complex community [62].

As first, TasA was considered as a protein with antimicrobial activity associated to spores [51]. In 2006, Branda and collaborators [35] found that TasA is the major protein component of the ECM in biofilms formed by *B. subtilis*. By co-culturing a *tasA* deficient strain together with a strain deficient in *eps* expression it shows that the TasA and the exopolysaccharides are shared by the bacteria within a biofilm, and the two deficient strains were able to form together a complemented biofilm [35]. In 2010, Romero and collaborators were able to determine the TasA structure as amyloid-like fibers, corresponding to a fiber conformation only when stimulated by hydrophobic surfaces [52] or in an acidic environment [34]. The secondary structure of TasA is dependent on the states, corresponding to an alpha-helix rich in the oligomeric state whereas in the fiber state are privileged the beta sheet structures [34].

B. subtilis spores

To survive periods of starvation *B. subtilis* can form dormant endospores. The spores can survive in this dormant state for many years [63] or even for millions of years as suggested by Cano et al. [64]. For the formation of an endospore, a *B. subtilis* single cell divides asymmetrically. As depicted in **Figure 2** in the "asymmetric septation" phase the two generated daughter cells are morphologically different [54,65,66]. The large and the small daughter cells are called mother cell and forespore, respectively. The mother cell, which

held to the forespore by the external cell wall, starts to engulf the forespore generating a double membrane inside its cytoplasm [67]. Finally, the formed spore partially dehydrates and develops into a dormant spore that is highly resistant to harsh environmental conditions, whereas the mother cell undergoes programmed cell death [67,68].

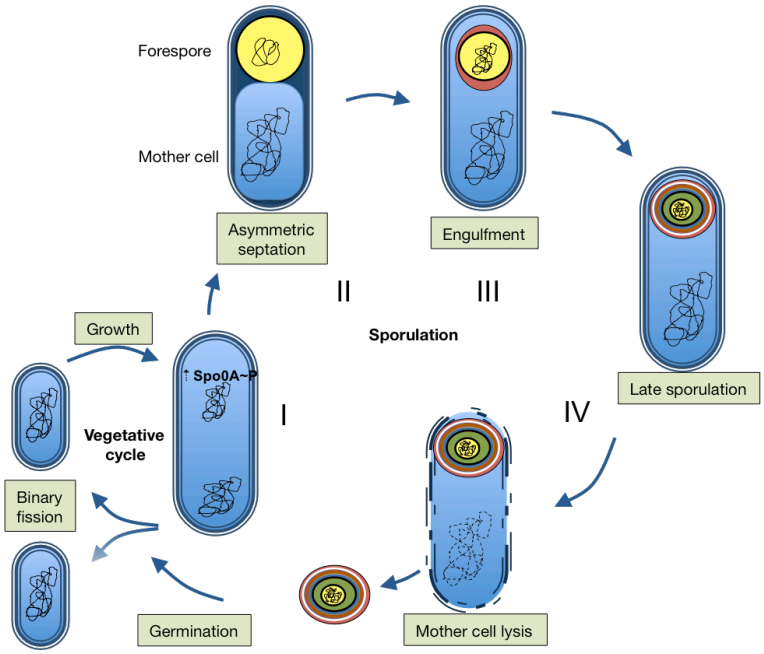


Figure 2: The stages of *B. subtilis* endospore assembly adapted from McKenney et al. **Stage 0)** Increasing amounts of phosphorylated Spo0A-P initiate the sporulation of the vegetative cells starting by the chromosome duplication. **Stage I)** Control of the number of chromosomes. **Stage II**) Asymmetric septation with temporary genetic asymmetry. **Stage III**) Engulfment of the forespore by the mother cell. **Stage IV**) Formation of the coat and the cortex, followed by the lysis of the mother cell and ending with the release of the endospore. In favorable conditions, the spore can germinate and go into a vegetative cell to start replication. Adapted from McKenney et al [79].

The sporulation process for *B. subtilis,* as illustrated in **Figure 2**, has been traditionally separated in four different stages ("I" to "IV"). However, these steps are not strictly separated and overlap each other [67,69]. The stages of the spore development and the three phases of the transition from the spore to the vegetative cell are described in detail below:

Spore differentiation stages (represented in Figure 2):

- **Stage 0: Initiation of the sporulation in the vegetative cell:** In a biofilm formed by *B. subtilis,* the sporulation does occur in subpopulations and not simultaneously for all cells [70]. The process for a single cell to start the sporulation is tightly regulated by the

phosphorylation of Spo0A. Lower levels of Spo0A~P lead to biofilm formation and extracellular matrix production, whereas higher levels of Spo0A~P lead to sporulation [55,71,72].

- **Stage I: Control of the chromosome copy number**. When the sporulation process starts, the cell contains two chromosomes aligned to each of the cell poles [73]. At the initiation of the replication, each chromosome interacts with the positioning control of the division site (DivIVA complex). In this manner, each new daughter cell receives one chromosome [67,74]. At least three different control proteins as SirA (inhibitor of replication in sporulation), Sda (dnaA1 suppressor) and Spo0A~P are involved in the correct allocation of the chromosomes [67]. The *sirA* is under the control of Spo0A~P and occurs upon sporulation is started. SirA is inhibiting the DNA replication by direct interaction with DnaA, which is inhibiting the binding and initiation at the origin of replication [75]. Additional to the indirect regulation of Spo0A~P it is a direct regulator of the DNA replication by its ability to bind to sites around the origin of replication, inhibiting DNA replication during sporulation [73]. The entry into sporulation is restricted to the period between rounds of DNA replication by Sda binding to the sporulation histidine kinase KinA during active DNA replication [67,76].

- **Stage II: Asymmetric septation.** In this stage, a temporary genetic asymmetry arises when the polar septum that separates the chromosome, includes only one-third of the chromosome in the forespore. The remaining chromosomal part is pumped by SpoIIIE, a DNA translocase, from the mother cell to the forespore [67,77].

- **Stage III: Engulfment**. In this stage, the mother cell engulfs the forespore by bending the polar septum around it, leading to a free floating cell in the cytosol of the mother cell [67].

- **Stage IV: Late sporulation and release of the spore.** In this final stage, the two shells, coat, and cortex, that protect the spore from environmental conditions are formed. The external shell (coat) and the inner shell (cortex) are composed of proteins and peptidoglycans [78,79]. Also, the coat consists of four layers: basement layer, inner coat, outer coat and the crust [79]. Thus, to release the spore the mother goes into a process of cell lysis and dies [67].

Transition phases from spore to vegetative cell:

The spores survive for long periods of unfavorable life conditions. As soon as the environmental conditions allow it, the spore gets reactivated.

- **Re-activation of the spore:** The re-activation of the spore triggered by environmental conditions as temperature, pH and chemical exposure leading to the so-called germination. Interestingly, this activation process can be reversible and does not necessarily lead to germination and outgrowth [80].
- **Germination:** Once the germination initiates, it becomes irreversible being unable to return to its dormant state [80,81]. Various stimuli can lead to germination, as are for example nutrients, ions, cationic surfactants and chelators [80,82].
- **Outgrowth:** This stage defined as all the events taking place after the germination concluded, which include the metabolism initiation, the macromolecules synthesis, the spore swelling, the emerge (outer spore layers shed) and the growth of the new cell [80].

The *B. subtilis* spore structure

In the case of *B. subtilis*, the spore is organized into eight main compartments shown in **Figure 3**, described from outside to inside: i) the crust, ii) the outer coat, iii) the inner coat, iv) the basement layer, v) the outer forespore membrane, vi) the cortex, vii) the inner forespore membrane and viii) the core containing the chromosome [25,79]. The spore coat is composed of several layers of more than 50 different proteins, responsible for the resistance to some chemicals and lytic enzymes but not to heat and radiation [25,83,84]. Most of these proteins are spore-specific and not found in the vegetative cell [85,86]. The outer forespore membrane is essential for the spore formation, but the spore resistance is not altered by modifications of the outer forespore membrane [66,84].

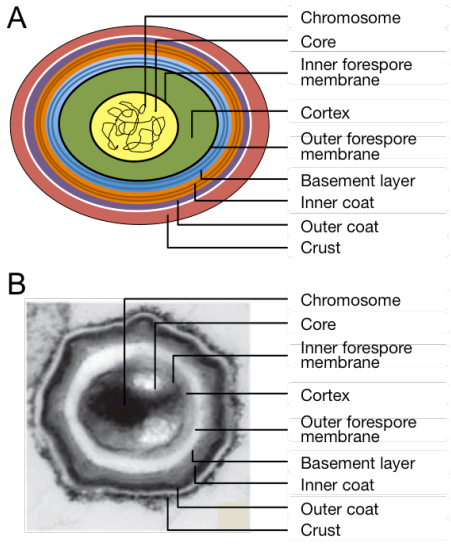


Figure 3. *B. subtilis* endospore representation adapted from McKenney et al. Schematic representation **(A)** and electron micrograph (**B**) of the *B. subtilis* endospore with the indicated structures. Adapted from McKenney et al [79].

The cortex of *B. subtilis* spores is mainly composed of peptidoglycans similar to those present in vegetative cells but has some spore specific modifications as the presence of

modified sugar muramic-∂-lactam and a low level of peptide cross-links between the glycan strands [87]. The cortex is essential in the spore, particularly for the reduction of the water content in the spore core. During the spore germination, the cortex is degraded, a process that is essential for the expansion of the spore core and the outgrowth [82].

The inner forespore membrane permeability provides the resistance to many chemicals, in particular, those damaging the chromosomal DNA [84,88]. In the dormant spore, the lipids of the inner forespore membrane are largely immobile, but they become fully mobile when the spore germinates [89]. The innermost structure corresponds to the spore core that contains the DNA, ribosomes, tRNA and enzymes, which are identical to those present in vegetative cells [25]. The low water content in the core is the major factor for the enzymatic dormancy and the resistance of the spore to moist heat [88]. Additionally, pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA), which found in about 5 – 15 % of the dry weight of the spore, has also been associated with resistance. The DPA exclusively locate in the core representing several functions as; i) chelating divalent cations, as Ca^{2+} [25], ii) reducing the water content during the sporulation and iii) a strong photosensitizer [85,90].

B. subtilis colonization of the gastrointestinal tract

B. subtilis spores were found to germinate in the jejunum and ileum of mice [91]. It is estimated that less than 1 % of the given spores *per oral* route to mice will germinate in the GIT [91,92]. A study with *B. subtilis*, spores denoted their full resistance to simulated gastric solutions. This study also showed that almost the same number of spores was recovered from the small intestine as orally administered to mice [93]. Two studies in chickens regarding the persistence of *B. subtilis* within the GIT showed the presence of spores in feces up to 36 days post-oral gavage [94,95]. In mice, the transit time in GIT for *B. subtilis* spores resulted to be 15 days [96]. Interestingly, an *in vitro* study that analyzed the interaction between recombinant and parental *B. subtilis* spores with human macrophages showed that *B. subtilis* spores get phagocytized at a frequency lower than 2.5 % and most of the internalized spores are killed by 6 hours [97].

Hoa et al. [98] found more spores shed than the number given to the mice by oral gavage, suggesting that spores were germinating and replicating in the GIT before re-sporulating and getting shed. Further proof going into the direction that *B. subtilis* is a gut commensal rather than an exclusive soil bacteria was found, when the team of S. Cutting isolated *B. subtilis* strains from human gastrointestinal tract [99].

B. subtilis use in oral immunization

The immunogenicity of *B. subtilis* spores was shown in the studies realized by the group of S. Cutting [15,100] using the Tetanus toxin fragment C as an antigenic model. In this case, the antigen was fused to the coat protein CotB. The recombinant spores were used to immunize mice that lead to protection against tetanus toxin in a challenge. However, as the protein is displayed on the surface of the spore, the presence of the fusion protein at the place of immunization after bypassing the stomach is uncertain [92]. Additionally, to the effect provided by the protein presented *B. subtilis* spores have already been used as probiotics in both humans and animals [101]. Probiotics are defined as live microbial feed supplements that can benefit the host by improving its intestinal balance [102], which include the competitive exclusion of pathogens [103] by competition for host mucosal receptor sites, secretion of antimicrobials, competition for essential nutrients and stimulation of host immune functions [103–106]. Also, *B. subtilis* can convert *in vitro* genotoxins to unreactive products [107]. In the specific case of *B. subtilis,* the probiotic effect is denoted by the immune stimulation of Peyer's Patches, mesenteric lymph nodes and primary lymphoid tissue from the gut associated lymphoid tissue (GALT) [100,108].

Antibiotics and the microbiota

The gastrointestinal tract of animals is populated by various bacterial communities, which are referred to as microbiota [109–111]. The interaction between the host and the microbiota is symbiotic, as the host provides a physical niche and nutrition for the microbiota and in retribution, the microbiota enhances the resistance of the host against infections and facilitates for some nutrients the absorption out of ingested food [112–115]. Moreover, the microbiota is essential for the development of the host's immune system and changes in the microbiota can have severe effects on the host as infections, atopy, arthritis, inflammatory bowel disease and diabetes [116–121].

The treatment with antibiotics leads to a fundamental change of the intestinal microbiota. It was shown in studies with volunteers that during the treatment one-third of the bacterial taxa changed and two years after the treatment the microflora was not reconstituted to the pre-treatment taxa [122–125]. Further led the antibiotics to a reduction of the GIT barrier function by changing the microflora, reducing intestinal Muc-2, and the decline of the immune defense by the reduction of RegIIIγ and reduction of $CD4^+$ T cells in the small intestine [126–130].

Gastro intestinal immunity

The GIT and its mucosal surface are fully competent in the generation of an innate and an adaptive immune response. The innate immune system provides cells for the uptake of antigens. Those cells after processing the antigens induce inflammation through secretion of pro-inflammatory signals. Whereas the adaptive immune system recognizes particular antigens, reacting specifically and generating immunological memory [131]. The dendritic cells (DCs) and the microfold cells (M) are responsible for the capture, the transport, the processing and the presentation of the antigens. In the GIT, the DCs have transepithelial dendrites that enable the cell to sample antigens directly from the lumen of the GIT [132]. The DCs can prime naïve B cells, which then cluster in the mucosal-associated lymphoid follicles forming germinal centers or, migrate from lymph nodes to the germinal centers. In here, the primed B cells clonally expand, differentiate and undergo to affinity maturation. The pre-mature B cells can migrate to the lamina propria and differentiate into polymeric IgA-secreting plasma cells by two mechanisms: i) through activation of $CD4^+$ T cells, DCs and the intestinal epithelial cells and ii) through a T cell-independent manner after direct activation of DCs loaded with commensal bacteria [133,134]. The polymeric sIgA are secreted as a dimer, providing it an enhanced binding capacity to the antigen. In the GIT lumen, the polymeric sIgA prevent the binding of the pathogens to the mucosal surface by building large complexes that enable to the mucus to trap the pathogens [135,136]. Furthermore, the sIgA can bind to pathogens that have bypassed the mucosal epithelium [135,137].

Oral immunization and malnutrition - experiences from poliovirus

The World Health Organization (WHO) used the oral poliovirus vaccine (OPV) in routine immunization throughout the polio eradication initiative. In industrialized countries with a single OPV dose the seroconversion rate was over 80%, and with three doses a protection close to 100% was reached [138]. However, the lower mean frequencies of response to any poliovirus serotype were reached for South India (40%) and Northern India (20%) [18,139,140]. Various hypotheses to explain these differences were raised including diarrhea cases, low zinc levels, low Vitamin A levels and malnutrition [141,142]. For example, in 2011 about 44% of the Pakistani children were reported to be malnourished, in correlating areas to the majority of cases of paralytic poliomyelitis [18,143]. A study conducted by Saleem et al. evaluated the efficacy of the vaccine depending on the nutritional status of the vaccinated children in a randomized control trial. They showed that among the malnourished children 13% were unprotected against polio

serotype 1, in which most of them suffered significantly more often of diarrhea in the seven days before the vaccination. However, this lack of immunity could be compensated by giving the combination of bivalent OPV + IPV which lead to protection against PV1 (97.6%), PV2 (96.1%) and PV3 (91.7%) in parity to the protection of normally nourished children who had not received IPV. Therefore, it was concluded that the malnutrition has a major impact on the poliovirus eradication and vaccination regime needs to adapt towards injected IPV vaccines additional to the OPV in areas with high prevalence of malnutrition [18].

The dog tapeworm *Echinococcus granulosus*

The dog tapeworm *Echinococcus granulosus* (*E. granulosus*) is the causative agent of cystic echinococcosis (CE) in humans and other intermediate or accidental hosts. According to the World Health Organization (WHO), this parasitosis is considered as neglected tropical disease [144]. Distributed nearly worldwide, but mainly prevalent in developing countries CE is responsible for considerable human morbidity, mortality and economic loss [145]. Only in the United States of America (USA), the economic impact of CE is about 2 billion US-Dollars per year, and the worldwide infected population has been estimated to be close to 3 million people [144,146,147]. The CE is caused by *E. granulosus* in its larval stage (metacestode), which is growing throughout the internal organs from the intermediate hosts (mostly ungulates like sheep and camels) and the accidental hosts (such as humans) forming cysts up to 20 cm in diameter [148]. Cyst formation particularly occurs in the liver and the lung, leading to the clinical signs of the disease [149]. Signs for a cyst located in the liver are abdominal pain, nausea, weight loss, general malaise and evidence of hepatic failure. If left untreated echinococcosis is progressive and fatal [150].

The life cycle of *E. granulosus*

In the *E. granulosus* life cycle, two kinds of hosts are involved (**Figure 4),** a definitive corresponding to the *Canidae* family as dogs and wolfs and an intermediate corresponding to ungulates as sheep and camels [151]. The final host shed the *E. granulosus* eggs in feces, which subsequently can get ingested by the intermediate hosts. In the stomach of intermediate hosts, the eggs hatch and the oncosphere are released in the intestine, where the oncospheres get activated [152]. After penetrating the intestinal epithelium and the lamina propria, the oncospheres are transported passively by the bloodstream or lymphatic fluid, reaching their final destinations, primarily the liver and

the lung. To protect itself the oncosphere develops into larvae, forming a hydatid cyst (metacestode) as shown in **Figure 4**. These unilocular fluid-filled bladders that develop in internal organs of the accidental and the intermediate hosts consist of two parasite-derived layers, an inner nucleated germinal layer and an outer acellular laminated layer (LL), which is surrounded by the host-derived fibrous capsule generated during the immune response [147,153]. The life cycle of *E. granulosus* closes when a definitive host eats the metacestodes of an intermediate host containing invaginated protoscolex. These settle in the small intestine after passing through the stomach fluid (pH 1.08 in fed and pH 2.05 in fasted dogs [154]). Once settled in the small intestine of definite hosts the protoscolex maturate, which take 4 to 5 weeks to reach sexual maturity. However, most canids do not show symptoms from the parasite [147].

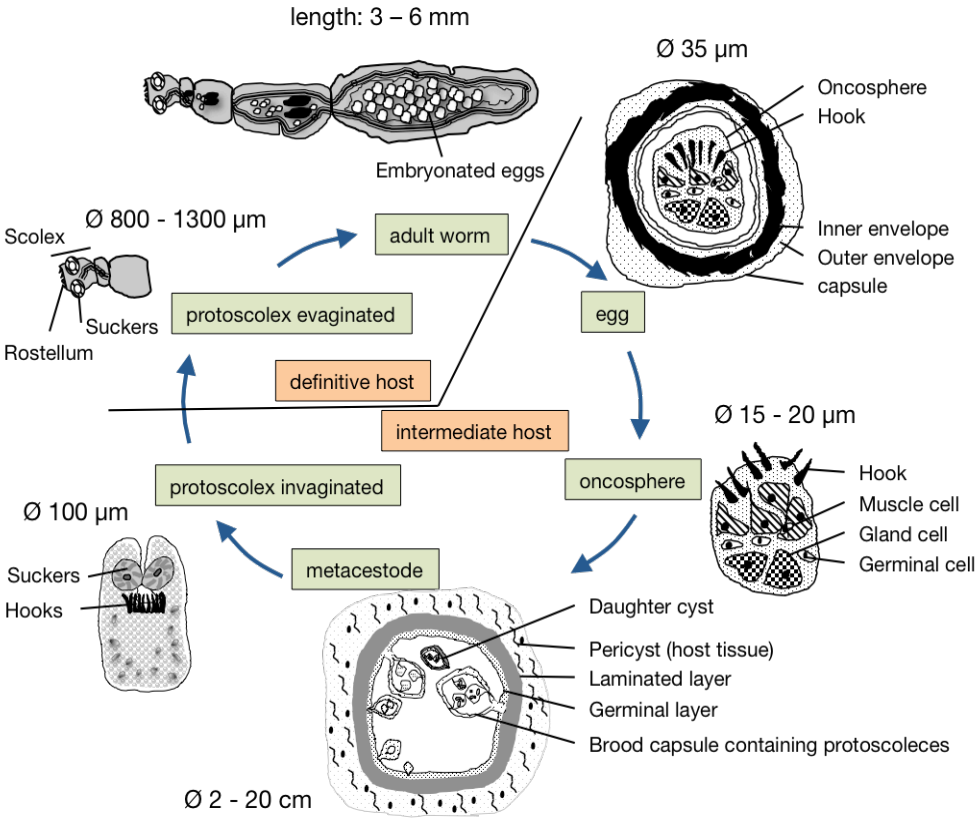


Figure 4. Schematic representation of the *E. granulosus* life cycle adapted from WHO/OIE.

The eggs, the oncospheres, the metacestodes and the invaginated protoscoleces are the forms of *E. granulosus* found in the intermediate hosts. The evaginated protoscoleces and the adult worms are found in the definitive host. The length and diameter of the structures are indicated. Adapted from WHO [150].

The immune escape of the metacestode in the intermediate host

The first barrier of the immune system from the intermediate host against the *E. granulosus* oncosphere is the barrier of the GIT. After penetrating the epithelium a significant cellular response is detected mainly based on eosinophils, lymphocytes and macrophages [147]. As consequence of the parasite implantation, the neutrophils and the macrophages infiltrate towards the *E. granulosus* oncosphere and mediate antibody-dependent cell-mediated cytotoxicity [155,156].

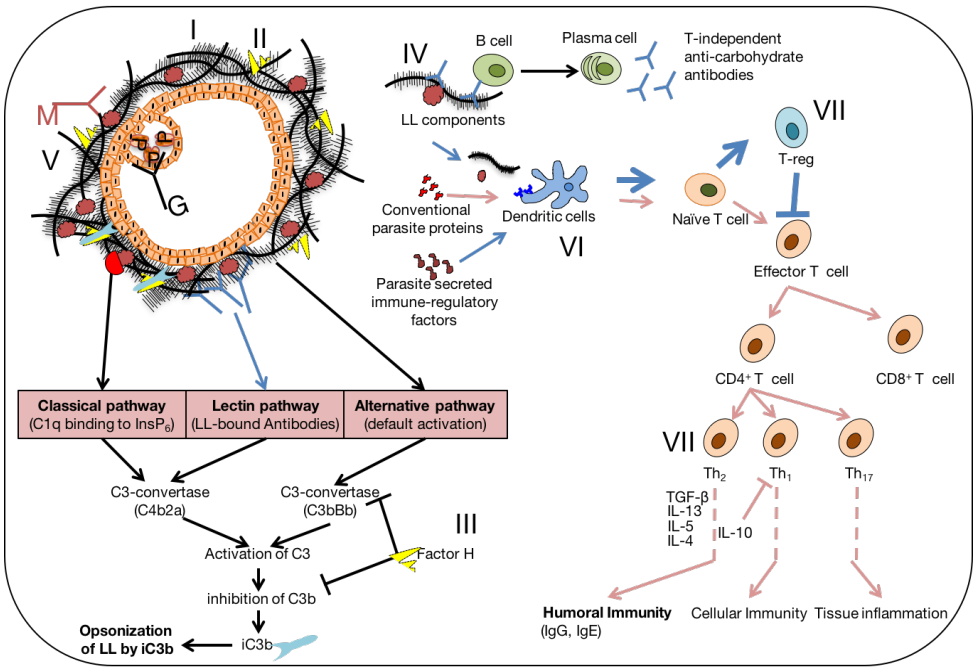


Figure 5. The human immune reaction against *E. granulosus* oncosphere and formed cyst adapted from WHO/OIE, Sjöberg et al, and Diaz et al. (I) The cyst laminated layer (LL) binds factor H **(II)** which is down regulating the complement system **(III)** by inhibiting C3b and its convertase (C3bBb). **(IV)** The LL components get recognized by B cells, which lead to its differentiation in plasmatic B cells resulting in the release of specific antibodies. **(V)** Whereas, specific IgM antibodies do not pass the LL but the IgG antibodies can penetrate the cyst. **(VI)** The parasitic proteins get screened by DCs. This screen is dependent on the ratio of activation factors (red arrows) and immune regulatory factors (blue arrows) secreted by the parasite. Immune regulatory factors lead to the formation of regulatory T cells **(VII)**, which inhibit effector T cells. The effector T cells develop depending on surrounding stimuli into Th2 cells **(VIII)**, that lead to a humoral immune response (IgG and IgE) effective against the parasite. Or into a Th1 cell response with a cellular immune response only partially effective and the Th17 cells which induce a tissue inflammation potentially inducing further anti-parasitic effects. Adapted from WHO [150,157,158].

However, as soon as the oncosphere develops into a metacestode and the hydatid cyst is

formed, the binding of Factor H of the complement system to the LL is inhibiting the complement system of the host shown in **Figure 5** [157,158]. Thus, the factor H is an inhibitor of the C3-convertase C3bBb, the C3b, and the C3bBbC3b C5-convertase. Through this inhibition, the LL gets covered by inhibited iC3b, which forms a physical barrier of host-self components between the *E. granulosus* tapeworm and the host immune system [157,158].

Further, the cells of the innate immunity as macrophages and mast cells lead to fibrosis, necrosis of cyst neighboring cells, vessel obstruction and bile duct [155,159]. Also, the eosinophil cells degranulate at the interface of the *Echinococcus* cyst and the host, whereas cationic proteins reach high levels in the hydatid cyst fluid that are harmful to the parasite [160]. Also, patients with a CE showed a significant number of NK cells in their peripheral blood mononuclear cells (PBMC) [161].

As a second strategy, the *E. granulosus* oncospheres infecting the intermediate hosts can increase the immune responses significantly, which include both the cellular and the humoral immune response [155]. In this case, the cellular immune response observed at an initial stage of the *metacestode* development led by a Th1 response that then shifts to a Th2 response in later chronic stages, characterized by high levels of IL-10 released by M2 macrophages involved in parasite elimination [162–164]. Nevertheless, *E. granulosus* can survive for many years in the intermediate hosts [165], due to the physical barrier of and by the cyst [166]. When a metacestode "leaks" from a ruptured cyst into other tissues or organs secondary cysts can establish [155]. A massive cellular infiltration towards the parasite takes place within three days, involving activated M2 macrophages, eosinophils and basophils and T regulatory cells that are recruited through chemokines [167,168], getting stimulated by parasitic immune modulators and evoking a Th2 response [147,169,170]. However, the *E. granulosus* tapeworm can control for most extend the hosts immune system as described in **Figure 5** and survives in the host's body [171].

<u>The immune escape of the adult *E. granulosus* in the definitive host</u>

The weak and ineffective immune reaction to a primary infection from *E. granulosus* to dogs leads to the hypothesis of immunomodulation by the parasite [172]. As described in **Figure 6** once the tapeworm gets implanted in the intestine of dogs by strongly adhering to the crypts of Lieberkühn it is in close contact with the gut mucosa[172]. Interestingly in a study performed by Rossi et al. [172] primary *E. granulosus* infected dogs developed a strong polarization towards a Th2 response. After this first infection with *E. granulosus* parasites, the dogs treated with the antihelmintic, praziquantel. Such infection and eradication cycles were repeated 3 and 6 times respectively. The results obtained

from this analysis showed the development of a balanced Th1/Th2 response with a following lower number of worms detected in the intestine of the dogs. Therefore, it seems to be helpful for the dogs to develop a Th1 driven immune response to reduce the *E. granulosus* worm counts.

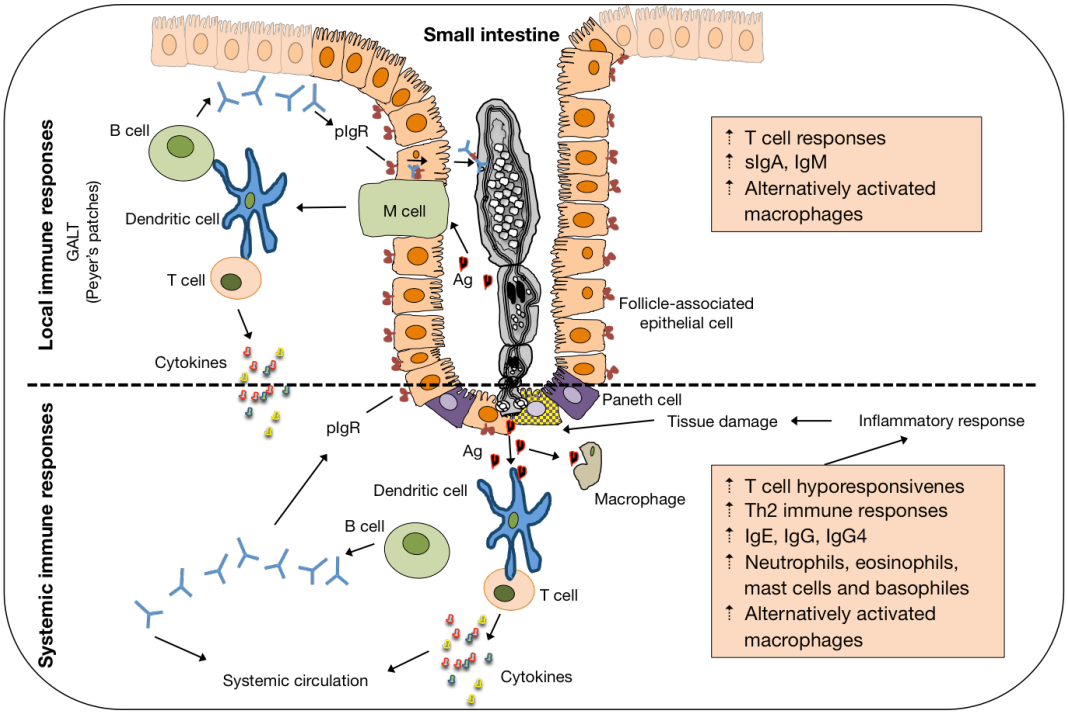


Figure 6. Schematic model of the immune reaction induced by *E. granulosus* in the dog infected small intestine adapted from Zhang et al. Antigens actively screened by M cells and DCs in the small intestine lumen. DCs process the antigens by then presenting them to the B cells, which can differentiate into plasmatic cells able to produce specific antibodies against *E. granulosus*. IgA transcytosis occurs in the epithelial cells by binding to the Ig-Receptor. The IgA are exported into the small intestine lumen and are effective against the parasite. Alternatively, the T cells can be activated releasing pro-inflammatory cytokines able to attract neutrophils, eosinophils, basophils and mast cells inducing a local inflammation with limited effects against the *E. granulosus.* Adapted from Zhang et al [155].

The anti-*E. granulosus* vaccine

Then, by considering the following three main points as i) a necessity to protect the intermediate and accidental hosts from *E. granulosus* infection [144], ii) based on a mathematical model showing the efficacy of reducing the *E. granulosus* burden by stopping dogs to spread *E. granulosus* eggs [173] and iii) the ability of the dogs to develop an immune reaction against *E. granulosus* [172] could lead to the bases for the development of an effective vaccine targeting *E. granulosus* protoscoleces in the definitive host like dogs.

E. granulosus tropomyosin and paramyosin as identified antigens

Up to now, some *E. granulosus* antigens as tropomyosin and paramyosin have been identified as possible targets for the development of a vaccine [174]. In a first instance, Esteves et al. characterized the *E. granulosus* tropomyosin (EgTrp), showing its expression in the suckers and the subtegument of the protoscoleces but also in the muscle of the tapeworm [175]. The mammalian orthologous for tropomyosin correspond to four highly conserved genes that by alternative splicing can rise to over 40 isoforms [176]. However, Alvite et al. [177] had reported only three isoforms of tropomyosin in the *E. granulosus* generated by the splicing of a unique gene. Further tropomyosin was also identified as potent antigens and therefore considered as vaccine candidates [178–180].

Also, Mühlschlegel et al. [181], identified the *E. granulosus* paramyosin (EgA31) which consists of a 97 kDa protein, localized in the tegument and subtegumental parenchyma in the protoscoleces. However, also mainly localized in the region of the suckers where it rapidly accumulates at the time of the head evagination [181,182].

EgTrp and EgA31 successfully tested for antigenicity in dogs

In a trial performed, using *Salmonella typhimurium* expressing EgA31 and EgTrp, Pétavy and collaborators [13] were able to immunize dogs which lead to more than 70% reduction in the worm counts after a challenge infection with *E. granulosus* when compared with the non-vaccinated dogs. Further, the worms from vaccinated dogs were smaller and less developed when compared to the worms extracted from the non-vaccinated dogs. However, the systemic humoral immune response showed no difference between the immunized and non-immunized group [13].

Based on these results, EgTrp and EgA31 will be considered as antigens for the vaccine strategy.

PhD Project

AIM. The aim of this Ph.D. project is to investigate the possibility of using *B. subtilis* to immunize hosts against enteric infections through an intestinal establishment of *B. subtilis* biofilm. For this purpose, the extracellular matrix protein TasA will be fused with selected antigens, derived from the enteric tapeworm *E. granulosus*. Mice as a model and dogs as the major host of the tapeworm will then be inoculated with recombinant *B. subtilis* spores to characterize the development of immune reactions against *E. granulosus.*

WORKING HYPOTHESIS. With this PhD proposal, I intend to answer fundamental questions related to the development of enteric immunity due to oral administration of *B. subtilis* spores followed by formation of an intestinal biofilm as the main factor for immunization. The model target used for this purpose is the tapeworm *E. granulosus*. To achieve these goals, the spores from recombinant bacteria need to retain their tenacity, which is necessary to bypass the stomach. Then, they must maintain their ability to germinate and replicate in the gut. Finally, they need to express enough antigen of interest in an immunizing form to stimulate a local immune response. To address these issues, I have subdivided my work into the following steps, each of which can be tested by a formal hypothesis:

Hypothesis 1: Recombinant *B. subtilis* spores, compared to wild type spores, are not altered in their tenacity and shelf life at room temperature.
Hypothesis 2: Recombinant *B. subtilis* spores can bypass the stomach of mice and dogs.
Hypothesis 3: Recombinant *B. subtilis* spores can germinate in the intestine of mice and dogs and can express the TasA-antigen fusion protein, forming an intestinal biofilm.
Hypothesis 4: Mice and Dogs develop, after oral administration of recombinant *B. subtilis* spores, a specific humoral and cellular immune response against selected *E. granulosus* antigens (EgTrp and EgA31) fused to TasA.
Hypothesis 5: The oral administration of combined dose of recombinant *B. subtilis* spores for EgTrp and EgA31 will induce an exacerbated humoral and cellular immune response.

Materials and Methods

B. subtilis strains

A list with the *B. subtilis* strains used in this study and its origins is showed in **Table 1**.

Table 1. *B. subtilis* strains used in this thesis

Strain	***Genotype***	***Reference/source***
168	Wild type domesticated	Kolter Lab. Harvard Medical School
NCIB3610	Wild type undomesticated	Kolter Lab, Harvard Medical School
ΔtasA (CA017)	tasA::Km^R	Vlamakis et al., 2008 [183]
ΔsinR (DS92)	sinR::Spc^R	Romero et al., 2010 [52]
ΔtasA/ΔsinR	tasA::Km^R; sinR::Spc^R	This study
TasA-mCherry(CA113)	AmyE::yqxM-sipW-tasA-mCherry ; Cm^R	Romero et al., 2010 [52]
ΔtasA/TasA-mCherry	tasA::Km^R; AmyE::yqxM-sipW-tasA-mCherry ; Cm^R	This study
ΔsinR/TasA-mCherry	sinR::Spc^R; AmyE::yqxM-sipW-tasA-mCherry ; Cm^R	This study
ΔtasA/ΔsinR/TasA-mCherry	tasA::Km^R; sinR::Spc^R; AmyE::yqxM-sipW-tasA-mCherry ; Cm^R	This study
ΔtasA/ΔsinR	tasA-sinR::Km^R	This study
ΔtasA/ΔsinR/TasA-(102-207)EgTrp	tasA-sinR::Km^R; AmyE::yqxM-sipW-tasA-(102-207)EgTrp; Spc^R	This study
ΔtasA/ΔsinR/TasA-(102-278)EgTrp	tasA-sinR::Km^R; AmyE::yqxM-sipW-tasA-(102-278)EgTrp; Spc^R	This study
ΔtasA/ΔsinR/TasA-(170-369)EgA31	tasA-sinR::Km^R; AmyE::yqxM-sipW-tasA-(170-369)EgA31; Spc^R	This study
ΔtasA/ΔsinR/TasA-(370-583)EgA31	tasA-sinR::Km^R; AmyE::yqxM-sipW-tasA-(370-583)EgA31; Spc^R	This study

Antibiotic concentration for *B. subtilis* strains

The final concentration of antibiotic used for the *B. subtilis* strains are the following: spectinomycin (Spc) (100 µg/ml), kanamycin (Km) (10 µg/ml), erythromycin (MLS) (1 µg/ml), lincomycin (25 µg/ml) and chloramphenicol (Cm) (5 µg/ml).

Plasmid constructions

The plasmids pDG-TasA-(102-207)EgTrp, pDG-TasA-(102-278)EgTrp, pDG-TasA-(170-369)EgA31 and pDG-TasA-(370-583)EgA31 were obtained by digestion of the plasmids pBS-TasA-(102-207)EgTrp, pBS-TasA-(102-278)EgTrp, pBS-TasA-(170-369)EgA31 and pBS-TasA-(370-583)EgA31 with XhoI and BamHI to obtain the fragments TasA-(102-207)EgTrp, TasA-(102-278)EgTrp, TasA-(170-369)EgA31 and TasA-(370-583)EgA31, respectively. The fragments were ligated into pDG1730 [184] between XhoI and BamHI restriction sites. The plasmids pBS-TasA-(102-207)EgTrp, pBS-TasA-(102-278)EgTrp, pBS-TasA(170-369)EgA31 and pBS-TasA-(370-583) were obtained by PCR amplification of EgTrpA and EgA31 fragments from the constructs pQIA-EgTrp and pQIA-EgA31 [185] using specific primers containing flanking NotI, BamHI and SmaI restriction sites, followed by ligation between NotI and SmaI in pBS-TasAop(SSSN). The plasmid pBS-TasAop(SSSN) was obtained by PCR amplification of TasAop fragment from pBS-TasA-mCherry [52] using specific primers containing XhoI and NotI restriction sites, followed by ligation between XhoI and NotI in pBS(SSSN). The plasmid pBS-MCS(SSSN) was obtained ligation of the annealed oligonucleotides (Fwd.: 5'-ATGCCTCGAGGGATCCTCAGAGTTAAATGGTA-TTGCT-3' and Rev.: 5'-GCATGCGGCCGCATTTTTATCCTCGCTATGCGC-3') between SalI and NotI restriction sites in pBluescript-KSII(+) (Stratagene).

The plasmid constructs pQE32-mCherry, pQE32-EgA31, pQE32-EgTrp and pQE32-TasA were obtained by PCR amplification of the fragments mCherry, EgA31 and EgTrp from pRSET-mCherry (Tsien lab), pQIA-EgA31, pQIA-EgTrp [175], and pET-TasA [52] using specific primers containing flanking BamHI and HindIII restriction sites and ligated in frame between BamHI and HindIII in pQE32 (Qiagen).

The long-flanking homology PCR (LFH–PCR) technique was used for creating deletion mutation, *tasA-sinR*::*Km*^{r} genes replacement, as described by Vlamakis et al., 2008 [183]. For this purpose, a joining PCR to create tasA-KmR-sinR was prepared from genomic DNA from *B. subtilis* NCIB3610 to amplify tasA with FtasAUP (5'-ACAATAAGTCATGGCCGGA-3') and RtasADO (5'-CCTATCACCTCAAATGG TTCGCTGGTTCGCTGGTTTAATACGCTGGCCAA-3') and sinR with FsinRUP (5'-

CGAGCGCCTACGAGGAATTTTATCGGCTCCC-CTTTTATTGAATG-3') and RsinRDO (5'-TATGCCGGCTATATGCTT-3'). The Km^R gene was obtained by amplification of genomic DNA from *B. subtilis* ΔtasA (CA017) strain [183] using the following primers FKmrUP (5'-CAGCGAACCATTT-GAGGTGATAGG-3') and RKmrDo (5'-CGATACAAATTCCTCGTAGGCGCT-CGG-3'). *B. subtilis* 168 tasA-sinR::Km^R was used as donor strain for transferring the mutant allele into the *B. subtilis* strain NCIB3610 using SPP1-mediated generalized transduction [186].

Oligonucleotides

The primers utilized for the construction of the different plasmids are signaled in **Table 2**.

Table 2: Primers used for plasmid construction.

Amplified segment	Oligonucleotide sequences
pBS-TasAop (SSSN)	Fwd.: 5'-ATGCCTCGAGGGATCCTCAGAGTTAAATGGTATTGCT-3' Rev.: 5'-GCAT*GCGGCC*GCATTTTTATCCTCGCTATGCGC-3'
pBS-TasA-(102-207)EgTrp	Fwd.: 5' ATGCGC*GGCCGC*CGAAACATCTACTAAGCTTGAC-3' Rev.: 5' GATCCCCCGGGGGGATCCTTACTCTTGCTCGGAGACTT GAG-3'
pBS-TasA-(102-278)EgTrp	Fwd.: 5' ATGCGCGGCCGCCGAAACATCTACTAAGCTTGAC-3' Rev.: 5' GATCCCCGGGGGATCCTCAGAAGGAAGTGAGCTCCGC-3'
pBS-TasA-(170-369)EgA31	Fwd.: 5' ATGCGCGGCCGCCGCAGCT5GAAAAACAAGCCATG-3' Rev.: 5' GATCCCCGGGGGATCCTCACCTTGTTTCAAGCATTTCAAT-3'
pBS-TasA-(370-583)EgA31	Fwd.: 5' ATGCGCGGCCGCCGCTGAGACTAAAGAAATTAAT-3' Rev.: 5' GATCCCCGGGGGATCCTCAATCTCTTTCGAGCTGTTTGAT-3'
pQE32-mCherry	Fwd.: 5' GATCGAATTCATGGTGAGCAAGGGCGAGGAG-3' Rev.: 5' GATCGCGGCCGCTTACTTGTACAGCTCGTCCATGCC-3'
pQE32-EgTrp-(102-278)	Fwd.: 5' GATCGGATCCTTGAAACATCTACTACTAAGCTTGAC-3' Rev.: 5' GATCCTGCAGTCAGAAGGAAGTGAGCTCCGC-3'
pQE32-EgA31	Fwd.: 5' ATGCGCGGCCGCCGCTGAGACTAAAGAAATTAAT-3' Rev.: 5' GATCCCCGGGGATCCTCATAATGCGTCCTTTTCTTTTTT-3'
pQE32-TasA	Fwd.: 5' CGAGCGCCTACGAGGAATTTGTATCGGCTCCCCTTTTATT AAT-3' Rev.: 5' TATGCCGGCTATAGCTT-3'

Transformation of *B. subtilis*

B. subtilis strain 168 was transformed routinely as follow [187], bacteria were inoculated in 10 ml of LB medium and grown overnight at 37°C with agitation. After centrifugation (2000 x g) the bacterial pellet was resuspended in transformation medium

(6 mM K_2HPO_4, 4 mM KH_2PO_4, 10 mM D-glucose, 100 mg/l casamino acids, 1.5 mM L-glutamate, 300 mM sodium citrate, 4 mM ferric ammonium citrate, 0.3 mM $MgSO_4$, 0.025 mM tryptophan and 0.025 mM phenylalanine) to $OD_{600\ nm}$ of 0.085 and let grow to early stationary phase until reaching $OD_{600\ nm}$ of 1.25. Then, 1 ml of bacteria were mixed with 5 ng of plasmid DNA, and the tubes were rolled for 40 min at 37°C. The cells were plated on LB agar medium supplemented with antibiotics. The transformants were selected with the appropriate antibiotics for a double crossover recombination at the amyE locus [184]. Thus, the positive clones were analyzed by direct PCR of the selected colonies. For this, each colony was resuspended in 15 µl lysis buffer (50 mM KCl, 0.1 % Tween20, 10 mM Tris-HCl pH 8.3) The PCR reaction was performed using the following primers for designated for pDG1730 plasmid constructions, pDG5'-F (5'-ATAATTTTAAATGTAAGCGTT-3') and pTasop-R (5'-CTGTAAAAGAAGC AAAAAAAAA-3'). The PCR was performed using the following ingredients in a total volume of 50 µl: 0.2 mM dNTPs, 150 µg of the forward and reverse primer, 0.3 µl Taq DNA polymerase, 1 µl DMSO, supplemented with ddH_2O. The PCR was performed under the following conditions: 5 min at 95°C followed by 40 cycles of 30 s 95°C, 60 s at 50°C and 90 s at 72°C and then followed by 10 min at 72°C and storage at 4°C until analysis by agarose electrophoresis. TapA operon-tasA-fusions were then transferred to NCIB3610 by SPP1-mediated generalized transduction [186]. Briefly, *B. subtilis* 168 was inoculated in 3 ml of TY media (tryptone (10 g/l), yeast extract (5 g/l), NaCl (5 g/l), 10 mM $MgSO_4$, 100 µM $MnSO_4$, pH 7.2) supplemented with antibiotics and incubated for 5 hours at 37°C. Then, 200 µl of the culture was mixed with 100 µl of diluted SPP1 phage in TY media (10^{-3}, 10^{-4} and 10^{-5}), incubated for 15 min at 37°C and mixed with 3 ml TY top agar (TY media, 0.5 % agar), poured over TY agar plates (TY media, 1.5 % agar) and incubated overnight at 37°C. The plaques were collected, filtered (0.45 µm) and stored at -80°C. Then, 30 µl SPP1 lysed use to transduce 1 ml recombinant donor *B. subtilis* 168 strain in 9 ml of TY media and incubated for 30 min at 37°C. Then, the culture was centrifuged for 7 min at 2000 x g, and the pellet was resuspended in 300 µl of TY media, seeded in LB-agar plates supplemented with antibiotics and incubated overnight at 37°C. Positive clones were selected as described above. The recombinant *B. subtilis* strains were stored at -80˚C in 15% glycerol in LB media.

<u>*B. subtilis* biofilm formation</u>

For experiments using biofilms of *B. subtilis*, a single colony from semi-solid LB agar with selective antibiotics was resuspended in LB media with antibiotics and grown to an $OD_{600\ nm}$ to 1. Then, 2 µl were spotted on 25 ml MSgg (5 mM potassium phosphate

(pH 7), 100 mM morpholine propane sulfonic acid (MOPS; pH 7), 2 mM $MgCl_2$, 700 µM $CaCl_2$, 50 µM $MnCl_2$, 50 µM $FeCl_3$, 1 µM $ZnCl_2$, 2 µM thiamine, 0.5% glycerol, 0.5% glutamate, 50 µg/ml tryptophan, 50 µg/ml phenylalanine, and 50 µg/ml threonine, 1.5% agar [50]) semi-solid agar plates, let dry at RT and incubated at 30°C for the indicated times.

Quantification of fluorescence in *B. subtilis* biofilms

The strains *B. subtilis* wild type, TasA-mCherry, ΔtasA/TasA-mCherry, and ΔtasA/ΔsinR/TasA-mCherry were allowed to form biofilms as described above. At 24, 48, and 72 h post-inoculation, the biofilms fluorescence (photon/sec) and area (mm^2) were measured using Synergy HT, Biotek, Germany. The data correspond to the mean ± SEM from three different biofilms for each tested *B. subtilis* strain. The plot was performed using Microsoft Excel for MAC: 2011, version 14. 5. 8 software.

B. subtilis spore preparation

B. subtilis spores were prepared following an adapted protocol from Nicholson and Setlow [188]. Briefly, a colony of *B. subtilis* was inoculated in 3 ml LB media supplemented with antibiotics (spectinomycin (Spc) (100 µg/ml), kanamycin (Km) (10 µg/ml) and MLS: erythromycin (1 µg/ml) and lincomycin (25 µg/ml) and chloramphenicol (Cm) (5 µg/ml)) and incubated at 37°C for 18 h on a horizontal shaker at 180 rpm. The inoculum was expanded in 500 ml LB media in a 2 L Erlenmeyer flask and let grow for 12 h at 37˚C in a shaker at 180 rpm. Afterward, 50 ml of the culture were diluted in 500 ml Difco sporulation media (DSM) contained in a 2 L Erlenmeyer Flask (bacto nutrient broth 8 g/L, KCl 1 g/L and $MgSO_4$ 0.25 g/L, autoclaved and allow to cool down to 50°C. To 1 L of this solution, 1 ml of each of the following filter sterilized solutions were added: 1 M $Ca(NO_3)_2$, 10 mM $MnCl_2$ and 1 mM $FeSO_4$ [188]. As a routine, 4 L of DSM were prepared for each spore stock. The culture was incubated for 72 h at 37°C on a horizontal shaker at 180 rpm. The spores were harvested by centrifugation at 14'000 x g, for 10 min and 4˚C; further, the supernatant decanted and the pellet heated for 30 min at 80°C. The pellet was resuspended in ten volumes of 1 M KCl / 0.5 M NaCl and centrifuged at 14'000 x g, for 10 min and 4˚C. The supernatant was decanted and the pellet resuspended in ten volumes of 50 mM TrisCl pH 7.2 and lysozyme [50 µg/ml], incubated for 60 min at 37°C and centrifuged at 14'000 x g, for 10 min and 4˚C. The pellet was resuspended in ten volumes of 1 M NaCl and centrifuged at 14'000 x g, for 10 min and 4˚C. Next, two turns of centrifugation and pellet resuspension were washed in

ten volumes of deionized water. Finally, the pellet was resuspended in 3 ml deionized water and stored at -80°C until use. The spores were titrated by diluting 20 µl of spore resuspension in 180 µl PBS (AccuGene, Lonza) and further serially diluted from 10^{-1} to 10^{-12}. A 10 µl drop from each spore dilution was inoculated over an LB media agar with antibiotics plate and let disperse vertically in the dish. The plates were incubated for 18 hours at 37˚C, and the number of colonies for each dilution was determined. The spore titer was determined as the colony forming units (CFU) per ml and calculated using the following formula:

$$CFU/ml = (n° \, colonies \times dilution^{-1})/volume \, in \, ml$$

Determination of the sporulation ability from *B. subtilis* biofilm

The ability of recombinant *B. subtilis* to sporulate in a biofilm was determined as described by *Vlamakis et al., 2008* [183]. Briefly, *B. subtilis* cultured in LB media were diluted to $OD_{600\,nm}$ of 1, and 10 µl of the suspension (in duplicated) were inoculated over 2.5 ml of MSgg media in 12 well plates. The plates were incubated at room temperature with no agitation. Samples of cells were taken after 48 h and subjected to mild sonication conditions (10 sec at 14 m Amplitude) to obtain single cells. The optical density of each preparation was normalized to $OD_{600\,nm}$ of 1 after sonication. The normalized preparations were incubated for 20 min at 80°C to kill vegetative cells. The viable spore counts were determined by serial dilutions plated from the normalized preparation after the 80°C incubation.

Immunoblotting

Protein detection was performed by immunoblotting [189,190]. Biofilm in MSgg agar was harvested at 72 h into in 500 µl deionized water followed by homogenization by sonication. Samples were normalized to $OD_{600\,nm}$ to 1. Then, 20 µl of normalized sample was mixed with 5 µl sample buffer (8% SDS, 40% Glycerol, 200 mM Tris pH 6.8, 4% 2-mercaptoethanol, 0.4% Bromophenol blue) and heated for 5 minutes at 95°C. Samples were loaded on an SDS-polyacrylamide gel (SDS-PAGE) [191]. The proteins were transferred to the nitrocellulose membrane (Amersham Protran 0.45 µm NC, GE Healthcare, #10600002) at 200 mA for 2 h in a wet tank Trans-Blot® cell (BioRad) containing Laemmli transfer buffer (for 1 L, pH 8.3: 3 g Tris Base, 14.4 g Glycine and 200 ml ethanol in deionized H_2O). The membrane was blocked for 30 min at room

temperature in blocking buffer (PBS, + 5 % nonfat milk). The primary and secondary antibodies were incubated at the indicated dilutions (**Table 3**) in blocking buffer for 2 h at room temperature on a teeterboard. The membrane was washed three times with blocking buffer between the antibodies and developing. The membrane was cleared with PBS and developed by using ECL western developing solution (Pierce™ ECL Western Blotting Substrate, ThermoScientific) and films (Lumi-Films, Roche, #11 6666 657 001). The exposed films were then developed using an RGII Fuji X-ray film processor and scanned for figure preparation.

Table 3: List of antibodies

Antibody name	Dilution	Used in	Origin
Rabbit polyclonal anti-TasA	1:20'000	WB, ELISA	A.F. Pétavy, Lyon
Rabbit polyclonal anti-TasA	1:1000	IF	A.F. Pétavy, Lyon
Mouse polyclonal anti-EgTrp	1:4000	WB, ELISA	A.F. Pétavy, Lyon
Mouse polyclonal anti-EgA31	1:3000	WB, ELISA	A.F. Pétavy, Lyon
Rat anti-CD4 mouse conjugate to PE	1:800	FC	BioLegend, #116005
Rat anti-CD8 mouse conjugate to PE/CY7	1:600	FC	BioLegend, #100721
Rat anti-CD4 canine conjugate to PE/CY 7	1:800	FC	eBioscience, #25-5040-41
Rat anti-CD8 canine conjugate to PerCP-eFluor710	1:300	FC	eBioscience, #46-5080-41
Mouse anti-CD25 canine conjugated to FITC	1:800	FC	eBioscience, #11-0250-41
Goat anti-Rabbit Alexa 594	1:500	secondary AB IF	sigma, #A-11037
Goat anti-mouse IgA-HRP	1:10'000	secondary AB ELISA	sigma, #A-4789
Goat anti-rabbit IgG-HRP	1:10'000	secondary AB WB, ELISA	sigma, #A0545
Goat anti-canine IgG HRP	1:600	secondary AB ELISA	sigma, #A6792
Goat anti-canine IgA HRP	1:600	secondary AB ELISA	Bethyl Lab., #A40-104P

Preparation of recombinant proteins

His_6 tagged proteins were produced in *E. coli* M15[pREP14], as described by the manufacturer protocol. Briefly, pQE32 constructs were transformed into *E. coli* M15 [pREP14], the isolated colonies were inoculated in 3 ml LB media supplemented with 100 µg/ml ampicillin and incubated overnight in a shaker. After that 900 µl of fresh LB

media with 100 µg/ml ampicillin were incubated with 100 µl of the overnight culture for 30 min at 37°C. Then the protein expression was induced by 1 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG, for 4 h at 37°C). The bacteria were centrifuged (14'000 x g, 4°C) and the pellet was kept at 4°C and suspended in 6 ml freshly prepared lysis buffer (PBS, lysozyme 1 mg/ml, protease inhibitor (cOmplete® Roche, #04 693 159 001)). Then the suspension was incubated for 15 min on ice. After the incubation lauryl sarcosine (0.15 v/v %) and dithiothreitol (final concentration 5 mM) was added and the suspension was sonicated 6 times for 10 sec in ice. The lysate was centrifuged for 15 min and 14'000 x g at 4°C. The His_6-tagged protein was then extracted using the PerfectPro Ni-NTA Agarose (5 Prime, #2400000), 0.5 ml of PerfectPro Ni-NTA was equilibrated with 2.5 ml (5 vol) of equilibration buffer (20 mM imidazole in PBS) and centrifugated at 800 g for 2 min. The resin was loaded with the bacterial lysate and incubated for 2 h in a wheel at 4°C. The resin was washed once with 10 volumes of the following solutions: washing buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 0.1% Triton100, 20 mM Imidazole), washing buffer 2 (50 mM imidazole in washing buffer 1) and washing buffer 3 (100 mM imidazole in washing buffer 1). Each washing step was performed for 30 min in a turning wheel at 4°C. The His_6-tagged protein was eluted from the resin with 2 volumes of elution buffer (50 mM NaH_2PO_4, 400 mM NaCl, 0.1% Triton100, 250 mM Imidazole) for 1 h at 4°C. The purified protein was dialyzed using snake skin® (Dialysis Tubing, Thermo Scientific, #88242). Every 2 h, the dialysis buffer was changed by dialysis buffer 1 (50 mM NaH_2PO_4, 400 mM NaCl, 0.1% Triton 100), dialysis buffer 2 (50 mM NaH_2PO_4, 350 mM NaCl) and dialysis buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl) in 200 volumes and 4˚C. The dialyzed protein was stored in 30 % glycerol at 4°C. The purity and the concentration of the recombinant protein were assessed by Coomassie blue staining in SDS-PAGE and confirmed by immunoblot using specific antibodies.

The Coomassie blue stained SDS-PAGE were digitalized using the transilluminator (BioRad), and the bands were analyzed by densitometry using ImageJ64 (version 1.48v, NIH, USA). A calibration curve was performed using known amounts of BSA.

For further confirmation of the selected proteins (H_6-EgA31 and H_6-EgTrp) were analyzed by mass spectrometry at the functional genomics center of the University of Zurich.

Characterization of the spore stability

Shelf life of spores

B. subtilis spores were diluted in deionized water to 10^7 CFU/ml, aliquoted in 300 µl into screw lid tubes and stored at room temperature in the dark until analysis. A first aliquot was directly analyzed (sample day 0). For each measurement, an individual aliquot was used at the indicated time point (4, 8, 12, 16, 20 weeks). The CFU was determined as described above (*B. subtilis* spore preparation). The CFU for each time point was compared to the sample from day 0. The experiment was repeated three times independently, and an average number of CFU was determined.

Heat stability test for spores

B. subtilis spores were diluted in deionized water to 10^8 CFU/ml and aliquoted in 300 µl into screw lid tubes. The reference sample was not heat-treated and remained at room temperature for further analysis. The samples were heated 30 min to the following temperatures: 85°, 95°, 99°, 121°C. The spores serially diluted and CFUs were counted on semi-solid LB agar plates after incubation at 37˚C. The experiment was repeated three independent times, and an average of the percentage of germinated spores compared to the untreated samples was determined.

PH resistance test for spores

B. subtilis spores were diluted in deionized water to 10^5 CFU/ml and aliquoted in 300 µl into screw lid tubes. The reference sample was treated as the other samples but with PBS at pH 7. The samples were treated with different pH 3, 2 and 1. The spores were suspended in a phosphate buffered saline solution adapted by HCl to the indicated pH. For this, the samples were centrifuged for 5 min 10'000 x g, and the supernatant was removed. The samples were then resuspended in 500 µl of PBS HCl with the corresponding pH. The samples were kept for 2 h at 37°C. After the samples had been washed twice with 500 µl PBS and suspended in 300 µl PBS, the spores were serially diluted and determined the CFU on semi-solid LB agar plates. The pH stability was expressed as the percentage of CFU compared to the corresponding pH 7 sample. The experiment was repeated for three independent times and the average percentage of CFU compared to the samples treated at pH 7.

Transmission electron microscopy of spores

Freshly prepared *B. subtilis* spores were frozen by high-pressure freezing, freeze substituted (FS 7500, Boeckler Instruments) with acetone containing 0.25 % glutaraldehyde and 0.5 % osmium tetraoxide at temperatures between -30°C and + 2°C as described in detail by Wild et al. [192] and then embedded in epon. The sections of 50–60 nm thickness were stained with uranyl acetate and lead citrate. The samples were analyzed by the transmission electron microscope (CM12, Fei) equipped with a CCD camera (Ultrascan 1000, Gatan) at an acceleration voltage of 100 kV. The pictures were analyzed using Fiji software [193–196].

Biofilm immunohistochemistry

Biofilms were grown for 72 h in MSgg-agar media. The biofilm was excised from the agar vertically using a surgical blade. The biofilm was oriented in the paraffin embedding in a manner that later semi-thin cuts of the biofilm in the paraffin block were vertical cuts of the biofilm. The semi-thin cuts were mounted on glass slides and then deparaffinized using two changes in xylene, 5 min each followed by transfer of the slides to xylene: alcohol (1:1) for two changes of 3 min each. Then the slides were transferred to successive washes of 95%, 70% and 50% alcohol, rinsed in PBS and blocked in 5% BSA-PBS (Bovine serum albumin fraction V, AppliChem) in a humid chamber. The samples were incubated with 100 µl of the primary antibody (rabbit anti-tasA, 1:1000) for 90 min a humid chamber at room temperature. Next, the samples were washed twice for 3 min in PBS-0.025 % TritonX-100, followed by two washes of 3 min in PBS. Then, the samples were incubated with 100 µl of 300 nM DAPI (4',6-diamidino-2-phenylindole) for 10 min in a humid chamber at room temperature and washed for two times (3 min) using PBS. The samples were incubated with the secondary antibody diluted in 150 µl (anti-rabbit Alexa Fluor 488, 1:500 in PBS-BSA 1%) for 60 min in a humid chamber at room temperature. The samples were washed twice with PBS, mounted in Prolong Diamond (Molecular Probes) and covered by a cover glass-slide. The histological samples were analyzed within 6 h after mounting, and images were acquired using a fluorescent microscope (LEICA DMI 6000B).

Animal experiments

Mouse experiments

All the mouse experiments were performed according to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocols were approved by the Cantonal Veterinary Office with the following animal experimentation number 104/2012 (**Annex I**).

Schedule of mouse experiments

Mice were treated three times by oral gavage on days 1, 21 and 42 of the experiment. The experiments were terminated on day 50 through mice euthanasia by isoflurane sedation and neck dislocation. Mice were dosed intragastrically with 5 x 10^{10} spores diluted in a final volume of 0.2 ml of saline solution (PBS). Each mouse experiment had the following experimental groups: placebo (0.2 ml saline solution), background *B. subtilis* spores (ΔtasA/ΔsinR) and recombinant *B. subtilis* spores. Blood and feces samples were collected before each oral gavage and on day 50. The feces samples were collected for 6 days after the first immunization, during the antibiotics treatment and before each of the subsequent immunizations and in day 50. Blood samples were collected by tail bleeding the day before each immunization and at day 50. When terminating the experiments on day 50 T cells were isolated from the spleen, mesenteric lymph nodes, and Peyer's patches. During the experimental procedure, mice were grouped by six animals per cages, with food and drinking water *ad libitum*. The mice were kept exclusively in company with animals receiving the same treatment. For the collection of feces of a single mouse, animals were kept in single cages during the harvesting period. The mice health status was monitored by following their body weight during the experimental period.

Antibiotic treatment for microflora eradication

When indicated, mice were treated an antibiotic cocktail for the elimination of the gut microflora and performed as described by Shan et al.[197]. Briefly, the following antibiotics were diluted in the autoclaved drinking water: ampicillin [0.5 mg/ml] (Panreac), gentamycin [0.5 mg/ml] (Panreac), vancomycin [0.25 mg/ml] (Alfa Aesar), metronidazole [0.5 mg/ml] (Alfa Aesar), sucrose [20mg/ml] (Sigma). The duration and frequency of the antibiotics administration were set to 7 days for the first experiment and reduced for the further experiments to 3 days.

Blood analysis in mouse experiments

The blood was collected using micro trainer SST tubes (Beckton, Dickinson and Company, NJ USA) and processed according to the manufacturer's protocol. In brief, the blood was incubated at room temperature for 30 min to allow coagulation. The serum was separated from the clot by centrifugation (1000 x g, 3 min) and transferred into new tubes (1.5 mL Eppendorf) for storage at -20˚C until analysis.

Detection of specific humoral immune response by ELISA

Specific antibody content in blood serum was detected using indirect enzyme-linked immune absorbent assay (ELISA). Briefly, 100 ng of purified recombinant proteins histidine-tagged *Echinococcus granulosus* tropomyosin (H_6-EgTrp), histidine-tagged *Echinococcus granulosus* paramyosin (H_6-EgA31), histidine-tagged TasA (H_6-TasA), histidine-tagged mCherry (H_6-mCherry). Alternatively, homogenates ($OD_{600\ nm}$ = $2*10^{-4}$) of biofilms (72 h incubated) from recombinant *B. subtilis* strain ΔtasA/ΔsinR/TasA-(102-207)EgTrp and ΔtasA/ΔsinR/TasA-(370-583)EgA31 in coating buffer (30 mM Na_2CO_3, 70 mM $NaHCO_3$ adjusted to pH 9.6) were used to coat immune absorbent 96-well plates (MaxiSorp Polystyrene ThermoScientific) for 16 h at 4°C. Plates were washed three times with 400 µl PBS with 0.05 % Tween 20 and then blocked for 2 h with 200 µl blocking solution (1 % bovine serum albumin (BSA) in PBS). Blood serum (100 µl diluted to 1:100 in 1 % BSA-PBS) were incubated for 2 hours at 37°C in a humid chamber. Then, the plates incubated with 100 µl secondary antibody conjugated to peroxidase for 30 min at 37°C in a humid chamber (**Table 3**). After, each incubation with antibodies (primary and secondary) the plates were washed three times as described above. To reveal the plates using colorimetric substrate as is the TMB reagents (Pierce™ TMB Substrate Kit, ThermoFisher) by adding 50 µl per well. The colorimetric reaction was monitored by reading absorbance at $OD_{600\ nm}$, and the reaction was stopped when the highest value reached $OD_{600\ nm}$ to 1.5 with 50 µl H_2SO_4 1 M. The $OD_{450\ nm}$ was read out using a multi-spectrometer (SLT 340 ATTC Tecan). The obtained data were analyzed using Excel software (Mac2011, 14.5.8, Microsoft). The average value of two independent ELISA plates with the same loading was determined and plotted using Prism software (%.0d for Mac, GraphPad). For the statistical analysis see the corresponding section.

Detection of humoral immune response from mouse feces

Feces were collected over periods of 24 h from mice isolated in single cages and stored at -20°C until analysis. The feces were resuspended in PBS (5 ml PBS/ g feces) and homogenized 30 s by vortex to analyze the spore content and the specific IgA immune response. The resuspended samples were centrifuged (800 x g, 10 min) and the supernatant was recovered for further analysis. For the testing, the IgA content in feces, 360 µl of the supernatant was mixed with 40 µl feces buffer (1 % BSA, 0.01 % Triton100, 0.1 % 2-mercaptoehtanol and one tablet for each 7 ml PBS protease inhibitor (cOmplete™ EDTA-free protease inhibitor cocktail tablets, Roche)) and stored in ice. 100 ng of purified recombinant proteins H_6-EgTrp, H_6-EgA31 and H_6-TasA or homogenates ($OD_{600\ nm} = 2*10^{-4}$) of biofilms (incubated for 72 h) from *B. subtilis* strains TasA-(102-207)EgTrp or TasA-(370-583)EgA31 were used to coat immune absorbent plates, as described above. The plates were washed three times with 200 µl 0.05 % Tween 20 in PBS and blocked for 2h with 200 µl blocking solution (1% bovine serum albumin in PBS). 100 µl per well of feces supernatant were incubated for 2 hours at 37°C in a humid chamber. The plates were washed for three times as above and then incubated with 100 µl per well of goat anti-mouse IgA conjugated to peroxidase (Sigma, #A4789), diluted in 1:600 in 1% BSA-PBS, for 60 min at 37°C in a humid chamber. Plates were developed using TMB substrate and data analyzed as described above (Detection of specific humoral immune response by ELISA).

Spores from mouse feces analysis

To analyze the spore content in feces and to eliminate vegetative cells 300 µl of the feces supernatant were heated 20 min at 80°C. Then, samples were serially diluted and plated on selective semi-solid LB-agar Kanamycin as described by Vlamakis et al. [183]. The obtained colonies were counted for the two highest dilutions containing colonies. The following formula was used to determine the number of spores per feces weight in grams.

$$\frac{CFU}{feces\ [g]} = (n°\ colonies \times dilution^{-1})/feces\ [g]$$

T cell proliferation assay by CFSE dilution

For the isolation of T cells from fresh spleen and a mixture of mesenteric lymph nodes (MLN) and Peyer's patches (PP), the tissues were dissected in pieces and mashed through a cell strainer into a petri dish (100 µm, BD Falcon #352360) to obtain single cells suspension.

The isolated mixture of MLN and PP cells were negatively sorted using magnetic EasySep™ Mouse T Cell Isolation Kit (StemCells # 19851). Thus, negative cells for the following cell surface markers: CD11b, CD11c, CD19, CD24, CD25, CD44, CD45R, CD49b, TCRγ/δ, and TER119 were selected using the manufacturer's instructions. Briefly, the cells were diluted in RPMI 1640 media to 1 x 10^8 cells/ml, and 50 µl/ml of rat serum were added to reduce unspecific binding of the antibody cocktail. 100 µl of the cell suspension were added to 400 µl of RPMI1640 media. Next, the antibody cocktail was added (25 µl) and incubated for 7.5 min at room temperature. Subsequently, a depletion cocktail (25 µl) consisting of specific anti-mouse conjugated antibodies against the cell markers CD11b, CD11c, CD19, CD24, CD25, CD44, CD45R, CD49b, TCRγ/δ, and TER119 was added and incubated for 2.5 min at room temperature. Finally, the magnetic beads (50 µl) binding to the conjugated antibodies were added and incubated for 2.5 min and placed over a magnetic plate for to 2.5 min to accelerate sedimentation of non-T cells previous to the supernatant collection. Then, the cells obtained from the supernatant were washed once with 5 ml RPMI 1640 followed by two washes with 5 ml PBS. Each washing step included the cell suspension in the solution by pipetting 5 times the suspension up and down followed by the centrifugation (10 min at 800 x g) and the removal of the supernatant by pipette.

The spleen cells were washed once with 5 ml RPMI 1640 (Gibco) and density selected for T cells using a HistopaqueR 1077 (Sigma) density cushion. For that, 5 ml of cell suspension were loaded over 5 ml of HistopaqueR 1077 and centrifugated for 10 min at 800 x g with no brakes. The cells from the top fraction were collected and washed once with 5 ml RPMI 1640 and two times with 5 ml PBS.

The T cells isolated from spleen, MLN and PP were stained with 1 ml of 1 µM of Carboxyfluorescein succinimidyl ester (CFSE) in PBS for 10 min at 37°C. Afterward, the cells were washed three times. Suspension of the cells with 5 ml RPMI1640 10% FCS, penicillin (50 units/ml), and streptomycin (50 µg/ml) (Gibco) and centrifugation for 10 min at 800 x g. Then the cells were seeded in a 96 multiwell with low adherence U-bottom plates (Falcon) at a density of $5x10^4$ cells per well. Each experimental point was seeded in triplicate and stimulated with one of the following reagents: 50 ng/well of PMA, 100 ng/well of purified H_6-TasA, 100 ng/well of purified H_6-EgTrp or 100 ng/well H_6-EgA31. All the stimulating agents were diluted in cell culture medium (RPMI 1640, 10% FCS, penicillin (50 units/ml), and streptomycin (50 µg/ml)). The cells were incubated for four days (37°C, 5% CO_2) and then analyzed by flow cytometry as described below.

Flow cytometry

Previous to the acquisition, the proliferating T cells were stained with the surface markers anti-CD4 and anti-CD8 antibodies directly conjugated to PE and PE/CY7, respectively (**Table 3**) and incubated for 15 min at 4°C in the dark. The cells were transferred to 5 ml analysis tubes (Greiner Bio-one), washed with 2 ml of PBS, centrifuged for 10 min at 800 x g and resuspended in 500 µl of PBS. The samples were kept at 4°C until acquisition. Thus, the samples were acquired using a Gallios flow cytometer (Beckmann Coulter) with the following settings: Forward Scatter Excitation (FSC) [(Ex): 488 nm, 862 Volt (V), gain 7.5]; Side Scatter (SSC) [Ex: 488 nm, 650 V, gain 2]; FL1 [Ex: 488 nm, 450 V, gain 1]; FL6 [Ex: 638 nm, 647 V, gain 1] and FL9 [Ex: 405 nm, 572 V, gain 1]. The obtained data were analyzed using the Kaluza analysis software (Beckmann Coulter) by setting gates in the manner to get single cell populations and that in the further gates the 99 % of negative control cells remained outside for the gated $CD4^+$ $CD8^+$ T cells populations. Thus, the gated cells were analyzed for proliferation monitoring CFSE signal intensity.

$$Percentage\ of\ proliferatingcells = \frac{sample\ proliferating\ cells}{PMA\ stimulated\ proliferating\ cells} * 100$$

So then the result plotted in Prism (5.0d for Mac, GraphPad).

Statistics of mouse experiments

Statistical analyses were performed by Prism software (5.0d for Mac, GraphPad) or Microsoft®Excel for Mac 2011, version 14.6.0 where indicated. The statistical differences with p-value < 0.05 were considered as significant. For the ELISA analysis, a Friedman-Test was applied followed by a Dunns post-test in inner group comparisons. Also, Kruskal-Wallis-Test followed by a Dunns post-test was used when the mean of the same time point but two different groups were compared.

The statistical analyses for body weight of the mice and the cellular immune response were obtained using one-way ANOVA followed by Tukey's post-test multiple comparison tests from Prism software.

Dog experiments

All the dog experiments were performed according to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocols were approved by the Cantonal Veterinary Office with the following animal experimentation number 100/2010 (**Annex II**).

Schedule of dog experiments

The dogs were immunized through oral administration with recombinant spores of *B. subtilis* on days: 1, 21 and 42. Blood samples were collected weekly starting one day before the first oral gavage. A total of eight animals were included in this experiment. The animals were euthanized on day 50 for the collection of the spleen, the mesenteric lymph nodes, the Peyer's patches, the intestinal fractions, and the intestinal content. For identification, the animals were tagged before weaning through the implantation of a transponder, as denoted by their identification number showed in **Table 4**. The dogs were kept in groups of three animals per cage. During the whole experimental procedure, the animals were provided water and food in amounts appropriated to their age and size. According to the Swiss schedule for dogs vaccination, all the dogs were vaccinated at the age of 13 weeks against leptospirosis, canine distemper, canine hepatitis, parvovirus infection and parainfluenza viruses (Canigen L Virbac and Canigen SHA_2PPi).

The eight dogs were distributed in four experimental groups receiving (details in **Table 4**): i) placebo (saline solution), ii) $2.5x10^{10}$ CFU recombinant spores *B. subtilis* (102-207)EgTrp and $2.5x10^{10}$ CFU recombinant spores *B. subtilis* (102-278)EgTrp, iii) $5x10^{10}$ CFU recombinant spores *B. subtilis* (370-583)EgA31 and iv) mixture containing $2.5x10^{10}$ CFU recombinant spores *B. subtilis* (102-207)EgTrp and $2.5x10^{10}$ CFU recombinant spores *B. subtilis* (370-583)EgA31.

The dog experiment was performed in two separate rounds, having in the first round the two dogs receiving spores of the EgTrp strain and one of the placebo included. The remaining dogs were included in the second experiment round. This because of the insufficient number of dogs available at the first starting time point.

Table 4: Dogs identification numbers, gender, room localization and spores assignment

Transponder N°	Gender	Treatment	Room N°
756 0981 0061 6715	female	Placebo	1
756 0981 0061 7019	male	EgTrp	1
756 0981 0061 7005	female	EgTrp	1
756 0981 0055 6689	male	Placebo	2
756 0981 0055 6696	male	(370-583)EgA3	3
756 0981 0055 6674	female	(370-583)EgA3	2
756 0981 0055 6710	male	mix	3
756 0981 0055 6720	female	mix	2

Additionally, the dog's health status (body weight, body temperature, and general health signs as the gingiva, behavior and feces consistence) was monitored daily or weekly by the animal caretakers or a veterinarian physician, respectively.

Oral administration

The recombinant B. subtilis spores were diluted in 1 ml of cat milk (Whiskas® Mars, Virginia USA) to facilitate the dog swallow. The control group received only 1 ml of cat milk. The oral gavage with the *B. subtilis* spores was performed in morning hours after feeding the dogs.

Blood collection and purification of serum and peripheric lymphocytes

Blood samples were collected weekly from the jugular vein using CPT™ Vacutainer® blood collection tube (BD), according to manufacturer's instructions. Thus, permitting the separation of lymphocytes and monocytes from plasma and erythrocytes to be used for CFSE proliferation assay. Also, the collected plasma was used for detection of systemic humoral response and stored at -20°C until analysis.

Detection of specific humoral immune response by ELISA

For the analysis 100 ng of purified recombinant proteins H_6-EgTrp, H_6-EgA3, H_6-TasA, H_6-mCherry or homogenates ($OD_{600\,nm} = 2*10^{-4}$) of biofilms (72 h incubated) from recombinant *B. subtilis* strain ΔtasA/ΔsinR/TasA-(102-207)EgTrp and ΔtasA/ΔsinR/TasA-(370-583)EgA31 in coating buffer (30 mM Na_2CO_3, 70 mM $NaHCO_3$ adjusted to pH 9.6) were used to coat for 16 h at 4°C immune absorbent 96-well plates (MaxiSorp Polystyrene ThermoScientific). The plates were washed three times with 400 µl 0.05 % Tween 20 in PBS and blocked for 2 h with 200 µl of blocking solution (1 % BSA in PBS). Next, the blocking solution was discarded, and the wells were incubated

with 100 µl serum diluted 1:10 in blocking solution during 2 h at 37°C in a humid chamber. The plates were washed three times with 0.05 % tween20 in PBS and incubated with the anti-canine IgG HRP and anti-canine IgA HRP respectively (**Table 3**) for 60 min at 37°C in humid chamber. After washing three times the plates with 0.05 % tween20 in PBS, the ELISA was developed by adding 50 µl per well TMB substrate (Pierce™ TMB Substrate Kit, ThermoFisher). The colorimetric reaction was monitored by reading absorbance at $OD_{600\ nm}$, and the reaction was stopped when the highest values were reaching $OD_{600\ nm}$ to 1.5 with 50 µl H_2SO_4 1 M. The $OD_{450\ nm}$ was read out using a multi-spectrometer (SLT 340 ATTC Tecan). The data obtained were analyzed using Microsoft®Excel for Mac 2011, version 14.6.0. The background absorbance was subtracted from all values. An average was obtained for two independent identical plates for each of the measured samples. The PI for each animal was considered as a threshold and hence, subtracted from each measurement per dog. Only obtained values from plates coated with the recombinant proteins and the biofilm homogenate that were higher than those values obtained from plates coated with H_6-mCherry were considered as positives.

Dog necropsy

The dogs were euthanized by the veterinarian physician. The procedure was performed through intravenous administration of acepromazine 0.1 ml/10 kg (Prequillan™, AROVET AG) and second three overdosed of intravenous barbiturate (sodium pentobarbitone) at 80 mg/kg of dog [198].

The following samples were collected at necropsy: blood for the analysis of the cellular immune response analysis. Further samples of the duodenum, jejunum, ileum, cecum and colon were knotted using a thread in sections of 2 cm in length and knotted on both sides to prevent the loss of intestinal content. For each section of the intestine, one sample was frozen in liquid nitrogen and stored for further analysis at -80°C. Another sample was collected and stored in formalin 4% and further processed for paraffin semi-thin sectioning for histological analysis. The remaining parts of the small intestine of the two dogs receiving the mixtures of spores were flushed with PBS for analysis of the content of recombinant bacteria as described below. Samples from the spleen, MLN and Peyer's patches were collected into RPMI 1640 and kept on ice and processed for analysis within 3 hours.

Analysis of the intestinal content for the detection of recombinant Spores

The small intestine was dissected in duodenum, jejunum, and ileum. Each section was perfused by vigorous flushes with PBS to collect the intestinal content in a flask. Then, the intestinal content was centrifuged at 14'000 x g and 20°C for 15 min. The supernatant was discarded, and the pellet resuspended in 20 ml PBS and centrifuged at 1'500 x g for 10 min at 20˚C. The supernatants were collected and centrifuged at 14'000 x g for 15 min and 20˚C. The pellet was resuspended in 3 ml of PBS. Then, 20 µl of each suspension were used for serial dilution in PBS from 1 to 10^{-6}. 10 µl of each dilution were plated on selective semi-solid LB-kanamycin plates (10 µg/ml) and incubated for 36 h at 37°C. The colonies were counted, and the amount of CFU/mL was calculated as described in the mouse section.

To characterize the colonies isolated from the intestinal content specific PCR was performed. Thus, the colony was resuspended in 15 µl lysis buffer (50 mM KCl, 0.1 % Tween20, 10 mM Tris-HCl pH 8.3) and were heated to 99°C for 10 min. Next PCR mix (0.2 mM dNTPs, 150 ng of the forward and reverse primer, 0.3 µl Taq DNA polymerase, 1µl DMSO) was added over 5 µl of the lysate of the bacteria. The used internal primers were for detection *B. subtilis* recombinant EgTrp strains: forward 5'-ATGCGCGGCCGCCATTATGATGGCAATGAAATTG-3' and reverse 5'-GATCCCCGGGGGGATCCTTACTCTTGCTCGGAGACTTCGAG-3', and for *B. subtilis* recombinant strains EgA31: forward 5'-ATGCGCGGC CGCCGCAGCTGAAAAACAAGCCATG-3' and reverse 5'-GATCCCCGGGGG ATCCTCACCTTGTTTCAAGCATTTCAAT-3'. The PCR was performed with the following conditions: 5 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 52°C and 1 min at 68°C and conducted with 10 min of elongation at 68°C. The PCR samples were analyzed in an agarose electrophoretic gel for migration pattern.

T cell proliferation Assay

The tissue samples were dissected into pieces and passed through a cell strainer (100 µm, BD Falcon #352360) to obtain a single cell suspension. The cells were next density selected using a histopaque 1077 (Sigma) density cushion. The cell suspension was loaded over 5 ml of histopaque 1077 and centrifuged for 10 min at 800 x g without brakes. The top fraction above the histopaque 1077 was collected and twice washed using 7 ml RPMI 1640 media and centrifuged at 800 x g for 10 min. Then the pelleted cells were twice washed using 5 ml PBS 1x and centrifuged (800 x g for 10 min). Next, the cells were stained with 1 µM of Carboxyfluorescein succinimidyl ester (CFSE) in 2 ml

PBS for 10 min at 37°C in the dark. Then the cells were washed three times with 5 ml media (RPMI 1640, 10% FCS, penicillin (50 units/ml) and streptomycin (50µg/ml))and centrifuged at 800 x g for 10 min. The cells were seeded in a 96 multiwell with low adherence U-bottom plates (Falcon) at a density of $5x10^4$ cells per well. Each experimental point was seed in triplicate and stimulated, with 50 ng/well of PMA, 100 ng/well of purified H_6-TasA, 100 ng/well of purified H_6-EgTrp, 100 ng/well of purified H_6-EgA31 or 100 ng/well of purified H_6-mCherry diluted in cell culture medium (RPMI 1640, 10% FCS, penicillin (50 units/ml), and streptomycin (50 µg/ml). The cells were incubated for four days (37°C, 5% CO_2) and then analyzed by flow cytometry.
Blood-derived PBMC were collected weekly using the CPT Vacutainer. Followed by centrifugation at 1500 x g for 30 min. 1 ml of blood serum was removed before resuspending the PBMC. After the transfer of the suspension to a 15 ml plastic tube cells were centrifuged (800 x g, 10 min) and suspend using 5 ml of PBS. After another centrifugation (800 x g, 10 min) the cells were suspended in 1 ml PBS + CFSE (1 µM final concentration) and incubated for 10 min at 37°C. The cells were then washed three times with 5 ml RPMI1640, 10 % FCS, penicillin (50 units/ml), and streptomycin (50 µg/ml). Moreover, then the cells were platted in a U-bottom of low adherence plate with 5 x 10^4 cells per well. The blood-derived PBMC were stimulated and incubated as described above for the T cells derived from solid tissues of the dogs followed by flow cytometry.

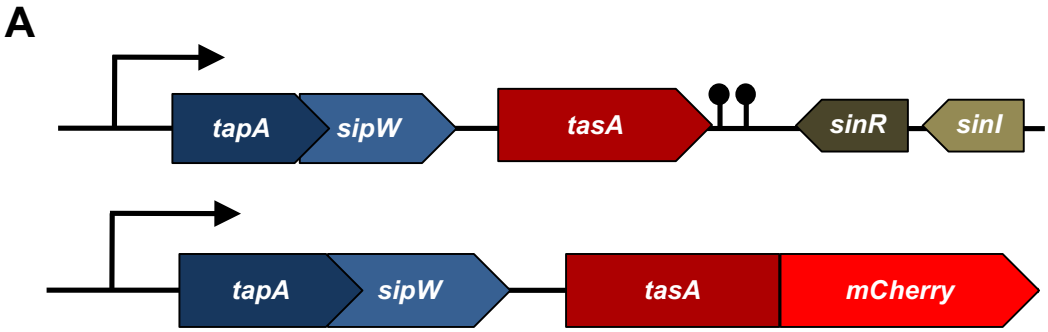
Previous to the acquisition, the stimulated cells were stained with the following surface markers anti-CD4, anti CD8, anti-CD25, anti-CTLA4 using FC antibodies (**Table 3**). For this, the cells were incubated for 10 min at 4°C in 5 ml analysis tubes (Greiner Bio-one) and kept at 4°C until acquisition. Thus, samples were acquired using a Gallios flow cytometer (Beckmann Coulter) with the following settings: Forward Scatter Excitation (FSC) [(Ex): 488 nm, 786 Volt (V), gain 2]; Side Scatter (SSC) [Ex: 488 nm, 817 V, gain 7.5]; FL1 [Ex: 488 nm, 368 V, gain 1]; FL2 [Ex: 488 nm, 443 V, gain 1] FL4 [Ex: 488 nm, 608 V, gain 1], FL5 [Ex: 488 nm, 807 V, gain 1], FL6 [Ex: 638 nm, 762 V, gain 1] and FL9 [Ex: 405 nm, 486 V, gain 1]. The obtained data were analyzed using the Kaluza analysis software (Beckmann Coulter) by setting gates to get single cell populations and that in the further gates the 99 % of negative control cells remained outside for the gated T cell populations. Thus, the gated cells were analyzed for proliferation monitoring CFSE signal intensity.

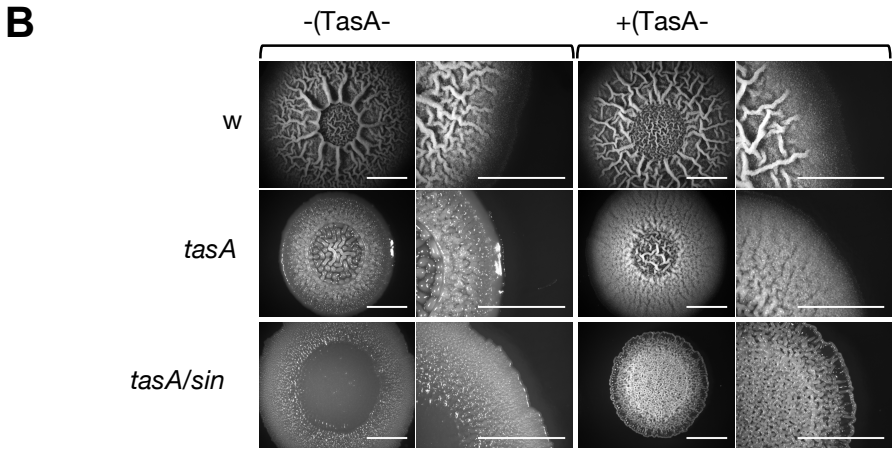
Results

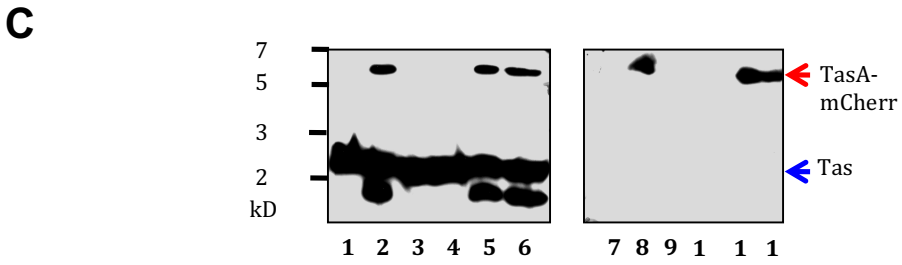
Parts of the results presented in this thesis were already published in Microbial Cell Factories journal and entitles as "Heterologous expression of antigenic peptides in *Bacillus subtilis* biofilms" [199] and a second manuscript based on the results presented in here, is currently in preparation.

Characterization of *B. subtilis* background mutant

One of the aims of this thesis was to express an antigen of interest in the biofilm of *B. subtilis* using a fusion of the antigen to the matrix protein TasA. As described above, TasA was observed to be homogeneously distributed in the biofilm, as visualized using a TasA-mCherry protein fusion [52]. The expression of TasA-mCherry in the biofilm was investigated using three different genetic backgrounds. First, in *B. subtilis* NCIB3610 (wild type, wt) [200]; second, in a deletion of the tasA gene (ΔTasA) [183] and third, in a double mutant for tasA and the repressor of the tapA promoter, the sinR gene (ΔtasA/ΔsinR). The whole TapA operon with TasA fused to mCherry (**Figure 7A**) was phage transduced into these three strains. The working hypothesis was that the TasA-mCherry fusion is functional as a component of the biofilm matrix, suggesting that TasA could be fused to an antigen of interest. To test this hypothesis, the phenotypes of 72 hpi biofilms from different strains (wt, ΔtasA, and ΔtasA/ΔsinR) were compared in the absence or the presence of TasA-mCherry. As depicted in **Figure 7B**, the TasA-mCherry fusion was able to partially complement the defective biofilms of the mutants ΔtasA and ΔtasA/ΔsinR, as observed by the worm-like structures and the wrinkles in the center and the edge of the biofilm. The expression of the TasA-mCherry fusion in the biofilm was monitored by immunoblotting (**Figure 7C**) using equivalent amounts of biofilm extracts and using anti-TasA or anti-dsRed (against mCherry) antibodies for detection. When incubating with anti-TasA, the biofilms formed by the strains transduced with TasA-mCherry (lanes 2, 5, 6, 8, 11 and 12) showed two bands of approximately 30 and 60 kDa, corresponding to the predicted molecular weights for TasA and TasA-mCherry, respectively.







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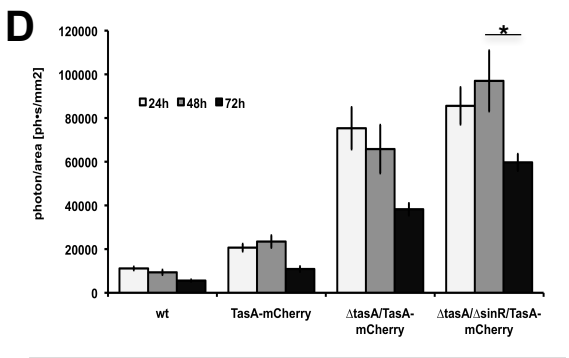
WB: anti-



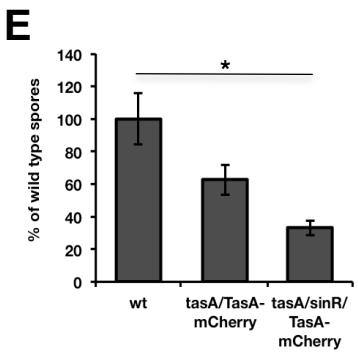


Figure 7. Comparison of the biofilm architecture and sporulation capacity of recombinant *B. subtilis* strains expressing the *TasA-mCherry* fusion.
(A) Schematic representation of the operon of tapA wild type and carrying tasA-mCherry. Black arrow; tapA promoter; the secretory protein (tapA); signal peptidase (sipW); extracellular matrix protein (tasA); TasA repressor (sinR); TasA inducer (sinI), the terminator (black pin). The mCherry gene represented by the red box replace the terminator, the SinR, and SinI gene. **(B)** Visualization of 72 h incubated biofilm grown on semi-solid MSgg-agar. Strains incubated are indicated, each either with –(TasA-mCherry) in the construct or without. **(C)** Immunoblotting of biofilm grown for 72 h. Homogenized biofilm ($OD_{600\ nm}$ = 1; 20 µl) of the strain wt (lanes 1, 3, 7, 9), ΔtasA/tasA-mCherry expressing TasA-mCherry (lanes 2, 5, 8, 11), ΔsinR (lane 4, 10), ΔsinR/ΔtasA/tasA-mCherry expressing TasA-mCherry (lanes 6, 12). The blots were analyzed with specific anti-TasA and anti-dsRed antibody respectively. The red arrow is indicating the TasA-mCherry fusion, and the blue arrow is indicating TasA. **(D)** The ability to express mCherry of indicated strains analyzed by analysis of the fluorescence of a biofilm at the indicated time points. (**E**) The sporulation ability of the indicated *B. subtilis* strains. Strains were inoculated for 48 h to develop biofilms before harvesting and analyzing the formed spores. Spore formation of the wild-type strain is shown as 100 %. The analyzed strains are indicated as the percentage of spores compared to the wild type strain. * ; p-value < 0.05.

A band of approximately 25 kDa was observed in samples expressing the TasA-mCherry fusion protein. As the band is a defined band, it was rather a by-product of the TasA-mCherry expression than a degradation product. Also, when incubating the membrane with anti-mCherry, it was possible to detect a unique band of 60 kDa corresponding to the predicted molecular weight for TasA-mCherry.

To elucidate which of the *B. subtilis* backgrounds showed the strongest expression of TasA-mCherry, the mCherry fluorescence (**Figure 7D**) was measured in the formed biofilms at 24, 48 and 72 h post-inoculation and normalized by the biofilm area. The results showed that the strongest expression of TasA-mCherry was obtained with the *B. subtilis* strain ΔtasA/ΔsinR/tasA-mCherry, displaying five times higher fluorescence than the *B. subtilis* wt background. Interestingly, the best expression of TasA-mCherry was obtained at 24 and 48 h post-inoculation, declining at 72 h post-inoculation. These temporal expression levels were explained by changes in the different cell-type populations in the biofilm community [183]. Since it was intended to administrate recombinant *B. subtilis* spores to the animals orally, we next investigated the ability of these strains to sporulate when compared to *B. subtilis* wt strain. For this purpose (**Figure 7E**), 48 h incubated biofilms from the *B. subtilis* strains ΔtasA/tasA-mCherry or ΔtasA/ΔsinR/tasA-mCherry both expressing TasA-mCherry, were harvested and the number of spores was determined and compared to the spores obtained from biofilms formed by *B. subtilis* wt. The results showed that a biofilm of *B. subtilis* ΔtasA/ΔsinR/tasA-mCherry had 25% of sporulation ability compared with a biofilm from *B. subtilis* wt but similar to the *B. subtilis* strain ΔtasA/tasA-mCherry. Even if reduced by percentage the number of spores obtained from ΔtasA/ΔsinR/tasA-mCherry showed only

a significant difference to the wt but not to the ΔtasA/tasA-mCherry strain. Therefore, the chosen background for the high expression of the desired antigen corresponded to the one provided by *B. subtilis* ΔtasA/ΔsinR.

The studies presented above were performed using a double mutant *B. subtilis* ΔtasA/ΔsinR obtained by replacement of the specific genes with two antibiotic resistances kanamycin and spectinomycin, respectively. Engineering of *B. subtilis* strains in a ΔtasA/ΔsinR background with the aim to facilitate the preparation was obtained by introducing a single antibiotic resistance gene for kanamycin. The biofilm developed by this newly engineered strain, hereafter named ΔtasA/ΔsinR, was phenotypically compared to the biofilms produced by *B. subtilis* wt, ΔtasA and ΔsinR strains (**Figure 8B**). The results indicated that the biofilm phenotype was, as expected, defective as well as similar to the one obtained in the *B. subtilis* ΔtasA/ΔsinR with the two separate antibiotic resistances from **Figure 7B**. The biofilm formed by ΔtasA/ΔsinR with a single antibiotic resistance gene for kanamycin was flattened and slimy in the edges, like the one brought by the ΔtasA, and with a worm like structuring central zone as the ΔsinR biofilm phenotype. The absence of TasA in *B. subtilis* ΔtasA and ΔtasA/ΔsinR with a single antibiotic resistance gene for kanamycin was assessed by immunoblotting using a specific anti-TasA antibody (**Figure 8C**, lanes 2 and 4). The results were then compared with the expression of TasA in biofilm extracts from *B. subtilis* strains wt and ΔsinR (**Figure 8C**, lanes 1 and 3). Based on the collected data, it was decided to continue using the *B. subtilis* strain ΔtasA/ΔsinR with a single antibiotic resistance gene for kanamycin as the background strain for the construction of a *B. subtilis* carrying TasA fused to an antigen of interest.

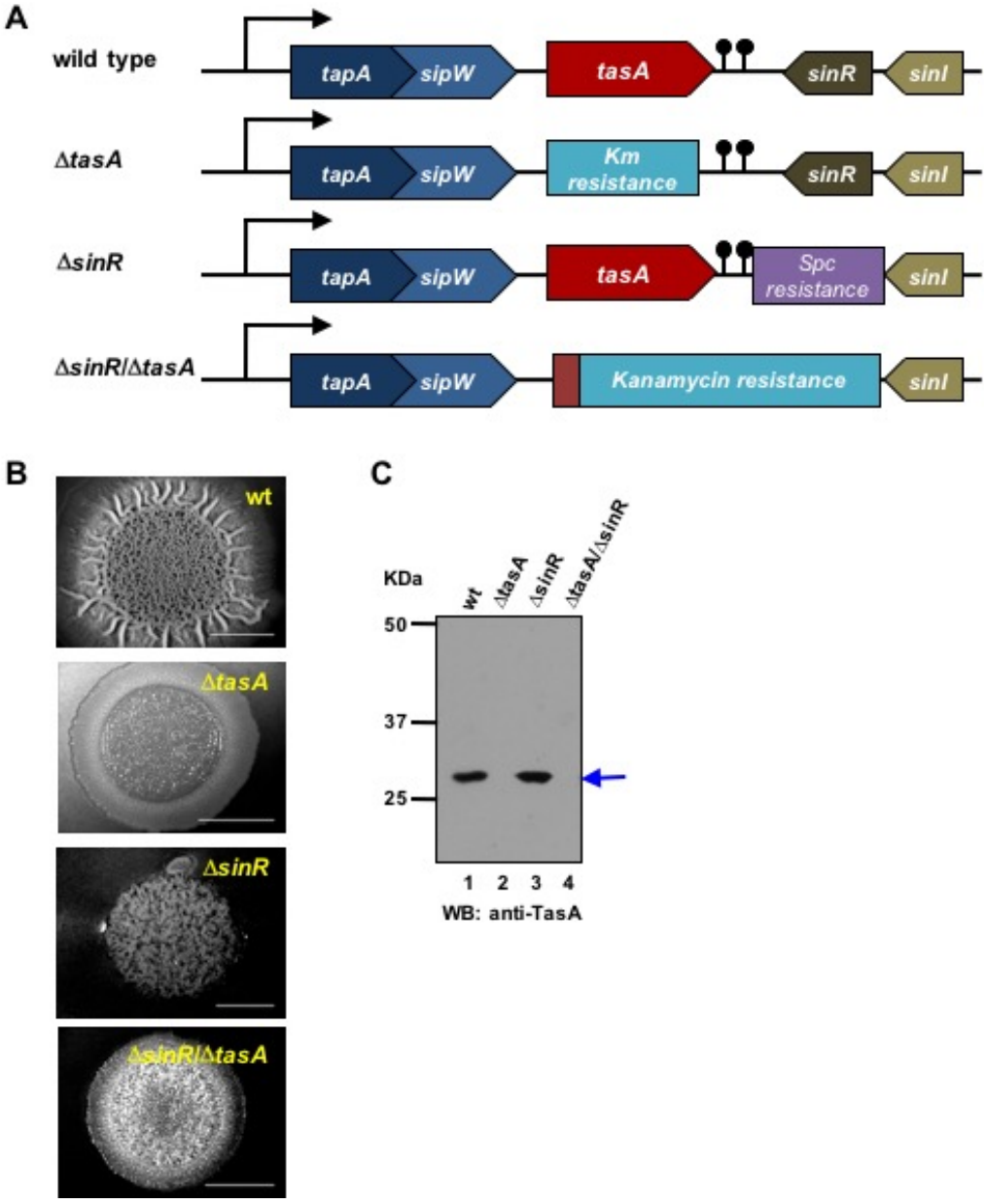


Figure 8 Characterization of the *B. subtilis* biofilm phenotype when deleted tasA or/ and sinR genes. (A) Schematic representation of the operon of tapA. black arrow: TapA promoter; Secretory protein (tapA); signal peptidase (sipW); extracellular matrix protein (tasA); TasA repressor (sinR); TasA inducer (sinI); the position of the antibiotics resistance is indicated as blue box (Kanamycin (Km)) and purple box (Spectinomycin (Spc))]; the terminator (black pins). **(B)** Biofilm top view of the indicated *B. subtilis* strains at 72 hpi on semi-solid MSgg. Scale bar, 5 mm. **(C)** Immunoblotting of biofilm extracts of indicated *B. subtilis* strains at 72 hpi. Bands were detected using a specific anti-TasA antibody stained for the TasA expression. The blue arrow indicates TasA. The molecular weights (kDa) of the proteins are indicated. The lanes are numbered corresponding to the loaded biofilm homogenate; *B. subtilis* wt (1), ΔtasA (2), ΔsinR (3), ΔtasA/ΔtasA (4).

Expression of TasA fused to tropomyosin *E. granulosus* antigen peptides in *B. subtilis* biofilms

To test the expression of *E. granulosus* tropomyosin peptides (EgTrp) in *B. subtilis*, various TasA-EgTrp fusions were phage transduced into the ΔtasA/ΔsinR *B. subtilis* strain. A schematic representation of the different constructs for the TasA-EgTrp fusions is depicted in **Figure 9A**. Two new recombinant *B. subtilis* strains were generated after that abbreviated as (102-207)EgTrp and (102-278)EgTrp, respectively. The recombinant operon was inserted by homologous recombination in the genome of *B. subtilis* by replacing the non-essential gene amyE (α-amylase) [201]. Clones growing on spectinomycin selective semi-solid media were tested for the phenotype of their formed biofilms. Biofilms showing a wrinkled and worm-like phenotype were selected (**Figure 9B**), homogenized, suspended and 10 µl of equal optical density was loaded in immunoblotting.

The immunoblotting (**Figure 9C**), when incubated with a specific anti-TasA (lanes 1 to 4) or anti-EgTrp (lanes 5 to 8) antibodies, showed a band of ≈ 30 kDa (lanes 1, 3 and 4) corresponding to the expected molecular weight of TasA. Also, it was possible to detect a band of ≈ 42 kDa (lanes 3 and 7) and one of the ≈ 48kDa (lanes 4 and 8) corresponding to the predicted molecular weight for the fusion protein TasA-(102-207)EgTrp and TasA-(102-278)EgTrp, respectively.

Based on its biofilm phenotypes and the detection in immunoblotting of the engineered TasA-EgTrp fusion proteins the two new *B. subtilis* strains, (102-207)EgTrp and (102-278)EgTrp we decide to include these two strains for further experimentation.

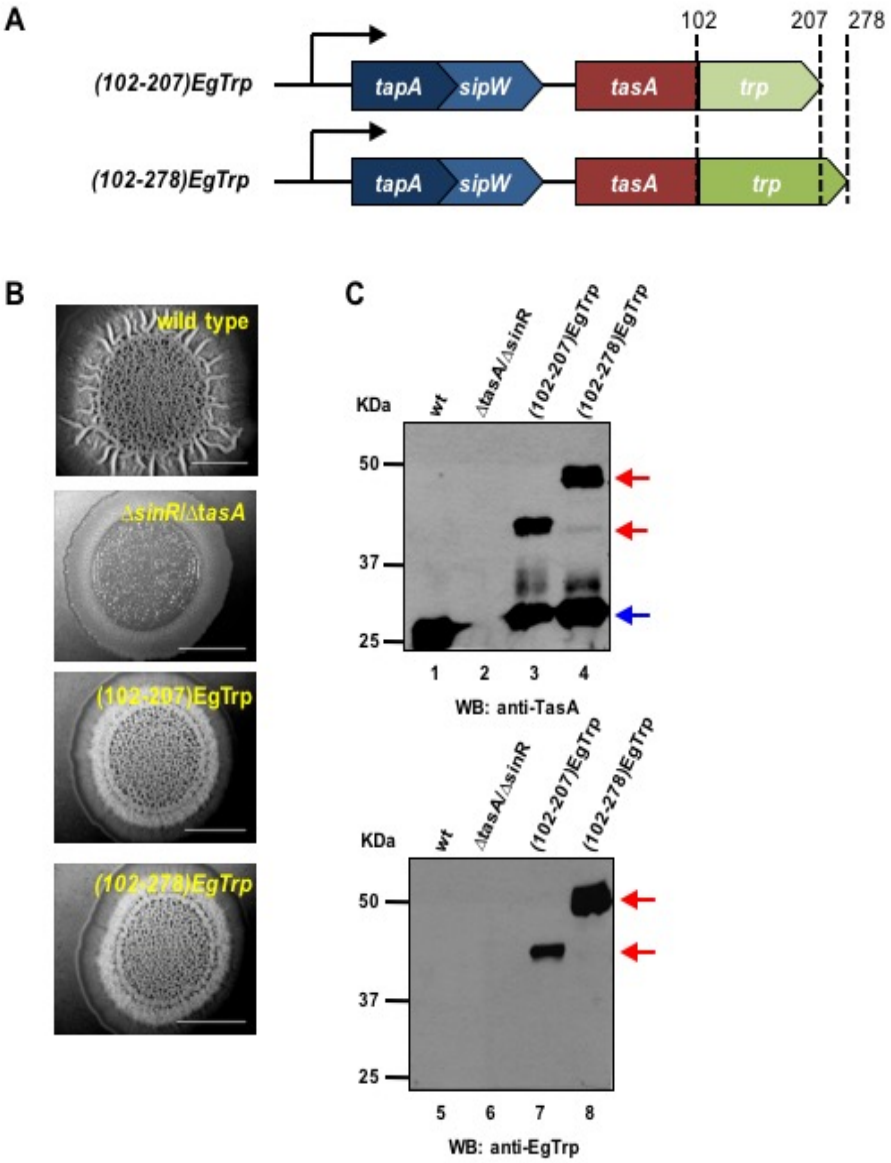


Figure 9. Expression of the fusion protein TasA-EgTrp in the biofilm of recombinant *B. subtilis* (102-207)EgTrp and (102-278)EgTrp strains.

(A) Schematic representation of the organization of the TasA-EgTrp operon transduced into the α-amilase locus of the *B. subtilis* ΔsinR/ΔtasA strain. Black arrow TapA promoter; secretory protein (tapA), signal peptidase (sipW); extracellular matrix protein (TasA); fusion protein (green arrow). **(B)** Top view pictures of 72 hpi biofilms of the indicated *B. subtilis* recombinant strains on semi-solid MSgg-agar. Scale bar, 5 mm. The incubated strains are indicated in the pictures. **(C)** Immunoblotting from homogenized biofilms at 72 hpi on semi-solid MSgg. The red and blue arrows indicate TasA-EgTrp fusion proteins and TasA protein, respectively. The protein molecular weights marker is indicated (kDa).

Expression of TasA fused to *E. granulosus* paramyosin antigenic peptides in *B. subtilis* biofilm

E. granulosus paramyosin (EgA31) was fused in frame to the C-terminus of TasA to test the expression in the *B. subtilis* deletion mutant (ΔtasA/ΔsinR). A scheme representing the engineered TasA-EgA31 fusion constructs is shown in **Figure 10A**. The main antigenic peptide for EgA31 (aa 170 to 583 [182]) was divided into two different amino acidic regions (170-369) and (370-583) and fused in frame to the C-terminal region of TasA. Analogous as described for the TasA-EgTrp strains (see also Material and Methods), two recombinant *B. subtilis* strains for TasA-EgA31 were generated named as (170-369)EgA31 and (370-583)EgA31, respectively. To determine the ability to form *in vitro* biofilms, the two recombinant *B. subtilis* strains, (170-369)EgA31 and (370-583)EgA31, were inoculated on semi-solid MSgg and let grow for 72 h as depicted in the **Figure 10B**. Under the tested conditions, the (370-583)EgA31 strain was forming structural complex biofilms with the central zone and wrinkles in the outer zone. Furthermore, the (170-369)EgA31 strain was forming a partially complemented biofilm showing fine worm-like structures in the central area and smooth wrinkles in the biofilm edges. Moreover, the presence of TasA-EgA31 antigenic peptides in biofilms extract was determined by immunoblotting of biofilms, using specific antibodies for TasA and EgA31. As depicted in **Figure 10C**, when incubating the membrane anti-TasA (lanes 1 to 6), it was possible to detect a smaller band of ≈ 30 kDa corresponding to the calculated molecular weight of TasA (lanes 2, 3, 5 and 6). In addition, a band of ≈ 50 kDa (lane 3) and one of ≈ 52 kDa (lanes 6 and 12) was detected when incubating with both anti-TasA and anti-EgA31, which corresponded to the predicted molecular weight for the fusions of TasA-(170-369)EgA31 and TasA-(370-583)EgA31, respectively. However, the strain (170-369)EgA31 was not expressing an amount of EgA31 detectable by the used anti-EgA31 antibody (lane 9) even having shown the expression in the analysis by the anti-TasA antibody (lane 3). Therefore the strain (170-369)EgA31 was excluded from further experiments.

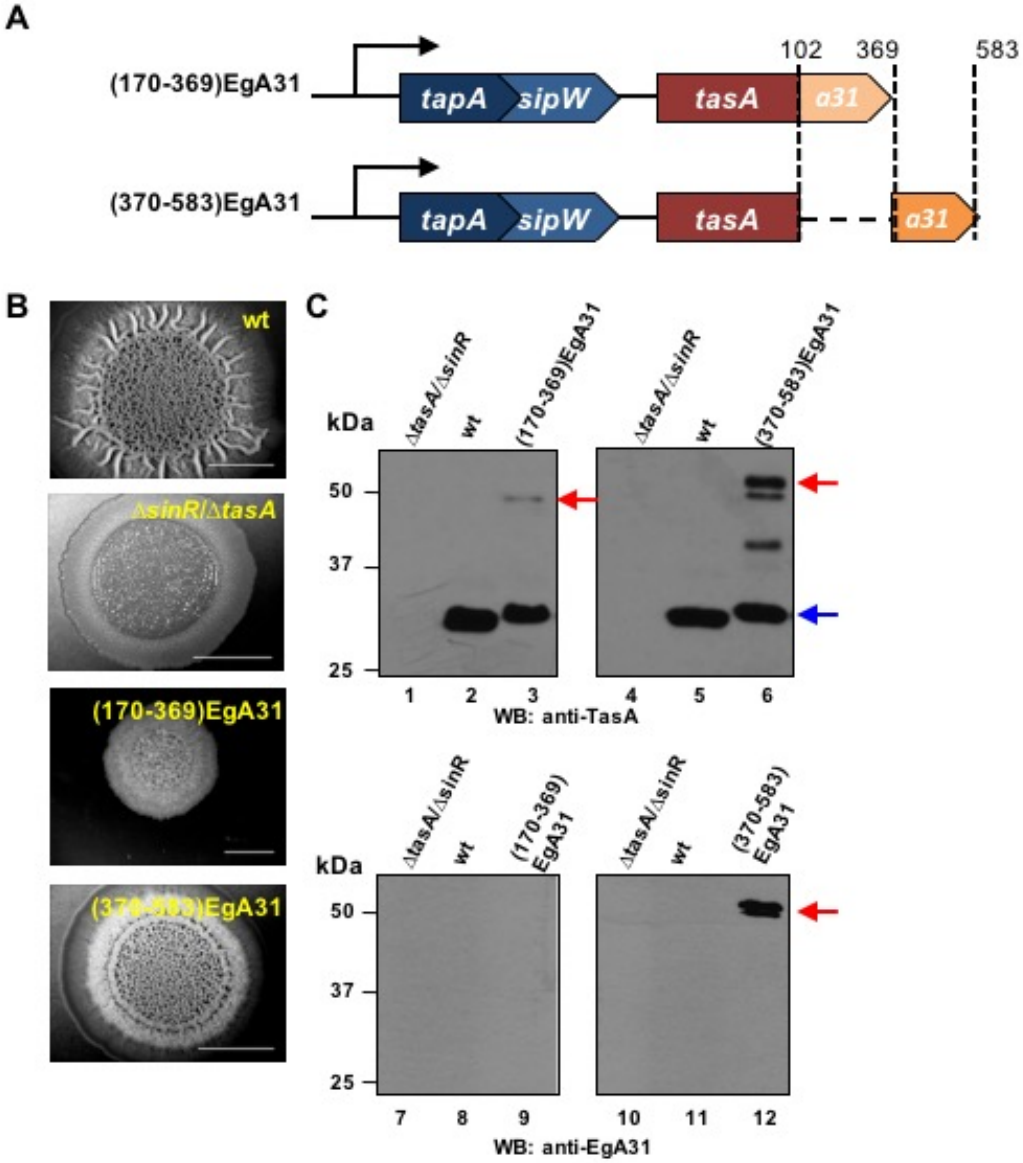


Figure 10. Expression of the fusion protein TasA-EgA31 in biofilm of recombinant *B. subtilis* (170-369)EgA31 and (370-583)EgA31 strains.

(A) Schematic representation of the organization of the TasA-EgA31 operon transduced into the α-amilase locus of the *B. subtilis* ΔsinR/ΔtasA strain. Black arrow TapA promoter; secretory protein (tapA), signal peptidase (sipW); extracellular matrix protein (TasA); fusion protein (green arrow). **(B)** Top view pictures of 72 hpi biofilms of the indicated *B. subtilis* recombinant strains on semi-solid MSgg-agar. Scale bar, 5 mm. **(C)** Immunoblotting from homogenized biofilms at 72 hpi on semi-solid MSgg. To detect TasA an anti-TasA and EgA31 an anti-EgA31 antibody were used. The red and blue arrows indicate TasA-EgA31 fusion proteins and TasA protein, respectively. The protein molecular weight marker is indicated (kDa).

Visualization of TasA in recombinant *B. subtilis* biofilms

To localize the fusion of TasA-*E. granulosus* antigenic peptides of the recombinant *B. subtilis* strains in their biofilms, an immunohistochemistry analysis was performed within 72 h post-inoculation. The biofilms were embedded and semi-thin vertical sections of the biofilms were prepared. Then, to localize TasA, the biofilm-sections were stained with a specific anti-TasA antibody followed by a secondary antibody conjugated to Alexa 594(red). As depicted in **Figure 11**, the obtained results showed that TasA fused to the antigen localize in the biofilm extracellular matrix.

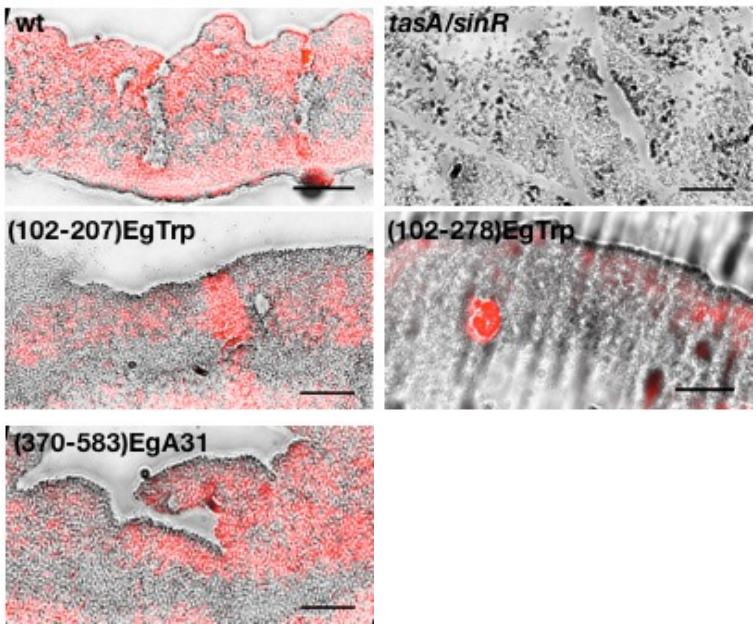


Figure 11. Immunohistochemistry of semi-thin paraffin cuts of 72 hpi biofilms from recombinant *B. subtilis* strains expressing EgTrp and EgA31 antigens, respectively. Vertical thin sections of biofilms. The name of each *B. subtilis* recombinant strain is indicated. Sections were stained for TasA (anti-TasA, red). Merge with the bright field. Scale bars, 50 µm.

Recombinant *B. subtilis* spores expressing *E. granulosus* antigens displayed unaltered heat, pH resistance, shelf life durability and spore structures

The resistance of the recombinant *B. subtilis* spores was analyzed upon four different parameters, as temperature, shelf-life viability, high pH and the structure of the spore outer layer and compared to the wt spores.

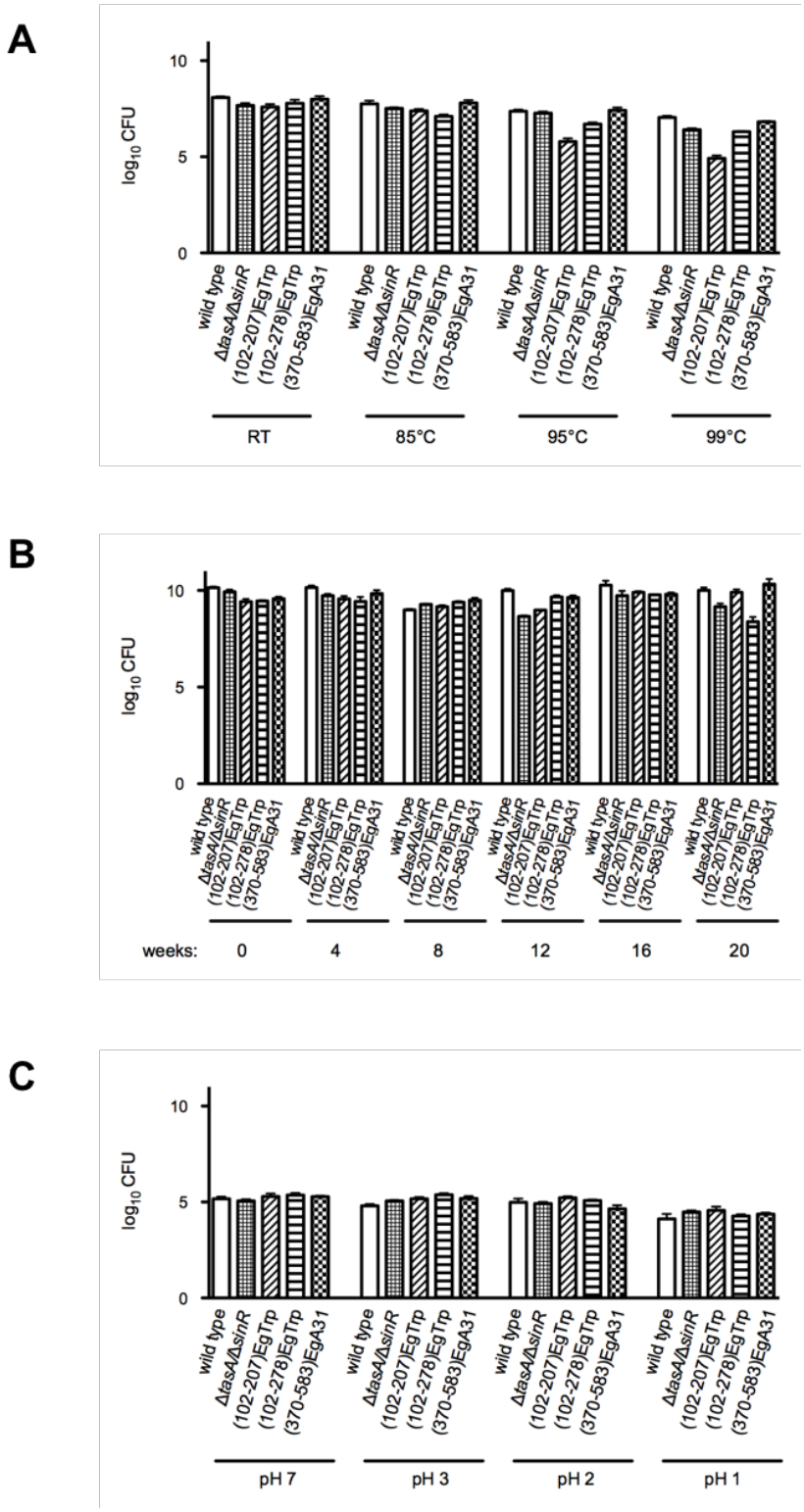


Figure 12. Recombinant *B. subtilis* spores exposed to different conditions.
(A) heat, the spores were incubated for 15 min at RT, 85°C, 95°C or 99°C, respectively. **(B)** shelf life store capacity in weeks at RT in the dark and **(C)** acidic pH variations. RT; room temperature (23°C) prior to inoculation on LB-agar to determine the CFU. Statistical analysis by one-way ANOVA, Tukey's multiple comparison test. Statistical significant is considered as a p-value < 0.05 for the differences between spores at a specific condition.

In a first instance, I compared the resistance of spores to different temperature by ranking from room temperature (23˚C) to 99˚C. The obtained results (**Figure 12A**) showed that all the tested recombinant spores had a similar amount of CFU and therefore a similar resistance to heat as the wild type spores. In addition, in the specific experimental setting, the recombinant spores got inactivated as the wild type spores did when treated with steam pressure for 30 min at 121°C.

Secondly (**Figure 12B**), I determined the shelf-life of the spores by analyzing the ability to germinate of the wild type and the recombinant spores after been stored in dark at room temperature for 0, 4, 8, 12, 16 and 20 weeks, which turned to be identical between wild type ant the tested recombinant *B. subtilis* spores.

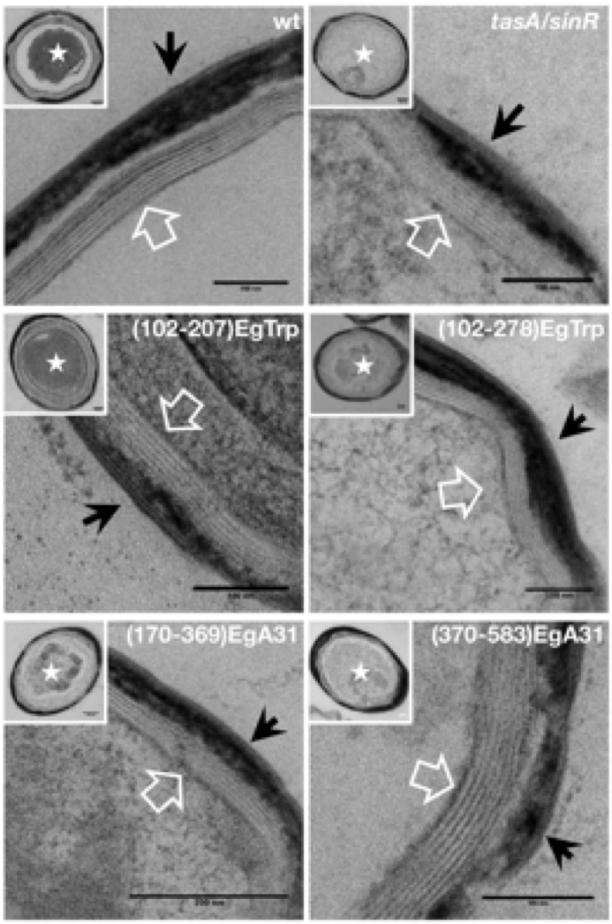


Figure 13. Transmission electron micrographs of freshly purified *B. subtilis* spores of the indicated strains.

Spores were frozen with liquid nitrogen, fixed with glutaraldehyde, counterstained and photographed. The star is marking the overview micrograph. In the magnification the open arrow is pointing the cortex peptidoglycan and the black arrow is pointing the spore

Third, the resistance of the spores in an acidic environment was tested. The spores were exposed for 2 h to different pH conditions as are pH 7, 3, 2 and 1. Further, the spores were washed, and the number of spores counted for each sample was compared to samples exposed to pH 7. The analysis of the obtained data showed that the recombinant spores were able to germinate in a number as the wild type spores did after being exposed to pH as low as pH 1 (**Figure 12C**).

Lastly, the purified spores from the recombinant *B. subtilis* strains were analyzed by transmission electron microscopy (TEM) for determining the outer structures of the spores, as are the outer coats and the inner cortex peptidoglycan. The recombinant

spores, visualized in **Figure 13**, were compared to the spores purified from the wild type *B. subtilis* NCIB3610. The outer coat appeared as thick electron dense layer surrounding the whole spore. When using higher magnification, a layered structure was observed corresponding to the outer coat. Also, I observed in the inner coat the multi-layered structures in the recombinant spores analyzed. The inner coat was less-electron dense than the outer coat and the layered structure was clearly defined. All compared spores showed a similar coat of peptidoglycans at the inner site of the layered structure, as well as a similar layered structure. Thus, all the *B. subtilis* spores, wild type and recombinant TasA-antigens, showed to be similar when compared by TEM in the structures of both the outer and the inner coats, analyzing for each indicated strain spores of one purification.

In conclusion, I stated that the generated recombinant *B. subtilis* mutants for (102-207)EgTrp, (102-278)EgTrp and (370-583)EgA31 when fused in frame to the C-terminal of TasA, were enabling the partial complementation of the defective *B. subtilis* ΔtasA/ΔsinR background by forming biofilm *in vitro* that were phenotypically similar to the *B. subtilis* NCIB3610 wild type. Additionally, as demonstrated by specific immunoblotting and immuno-histochemistry, TasA fused to *E. granulosus* antigens was located in the biofilm matrix. Lastly, I showed that the recombinant spores possessed the same ability to germinate as wild type spores after experiencing harsh conditions (temperature, shelf life and extreme pH) and as denoted by TEM showed a similar structure of organization of the outer most spore structures.

Based on the obtained results, I was able to generate recombinant strains of *B. subtilis* expressing antigenic peptides for *E. granulosus* when forming biofilms, while keeping the characteristic of the resistant spores formed.

Trial in mouse and dogs model to determine the capacity of recombinant *B. subtilis* spores to develop an enteric immune response.

A further aim of this Ph.D. thesis was to test the ability of the recombinant *B. subtilis* spores to generate specific cellular and humoral immune responses in mice and dogs. For this purpose, an immunization schedule was designed in which each animal (mice or dogs) was orally administered with recombinant *B. subtilis* spores carrying the antigen of interest for three times on days 1, 21 and 42. Due to its nature, it was hypothesized that the spores would be able to bypass the stomach barrier and be able to germinate in the small intestine of the inoculated animal. Thus, having the capacity to develop a biofilm, which would expose the antigens of interest. In this manner, the antigen could stimulate the gut-associated lymphoid tissue (GALT). In this context, it was also hypothesized that *E. granulosus antigens* (EgTrp and EgA31), when fused to TasA would be able to generate a specific humoral and cellular immune responses against the parasite. Therefore and based on the above-presented results, I decided to test the recombinant *B. subtilis* spores carrying the following *E. granulosus* antigens (102-207)EgTrp, (102-278)EgTrp and (370-583)EgA3 to investigate their ability to stimulate GALT in the gastrointestinal tract of both mice and dogs.

Evaluation of the ability of recombinant *B. subtilis* spores carrying (102-278)EgTrp and (102-207)EgTrp to induce humoral and cellular immune responses in mice

The mouse immunization schedule (**Figure 14A)** for the first mouse experiment, correspond to the oral gavage of 5 x 10^{10} CFU spores per dose on days 1, 21 and 42. In this trial, the following experimental groups were proposed: i) placebo (saline solution), ii) control spores (ΔtasA/ΔsinR), iii) recombinant spores of the (102-207)EgTrp strain and iv) recombinant spores of the (102-278)EgTrp strain. Each group contained six, six weeks old female Balb/c mice. Before each oral gavage and before euthanasia, blood and feces samples were collected to analyze the humoral immune response and quantifying the spores shed in the feces. On day 50, mice were euthanized and harvested mesenteric lymph nodes (MLN), Peyer's patches and spleen for studying the cellular immune response against EgTrp. The mice health was monitored by weighing them before each oral administration, on a daily base for seven days following the first oral gavage, and previous to each oral gavage and the euthanasia. As observed in the **Figure 14B**, the mice body weight development for all tested groups was unaltered, independently of the type of spores administrated. Thus, the data suggests that *B. subtilis* spores had no detrimental

effect on the mice health. Additionally, the ability of the spores to germinate after the passage of the GIT of the gavaged animals was determined. For this purpose, feces samples were collected from days 1 to 5 after the first immunization, before each oral gavage and the euthanasia. The obtained results (**Figure 14C**) show that the spores administered were shed in feces within 4 Days post-administration of the spores. Also, in the feces collected on day 21 and 42, from mice of the (102-207)EgTrp group contained spores, but on day 50 none of the animals were shedding spores. The total number of spores detected in feces was close to 10^9 CFU (**Figure 14D**), suggesting that a considerable number of spores get retained in the gastrointestinal tract of the mice.

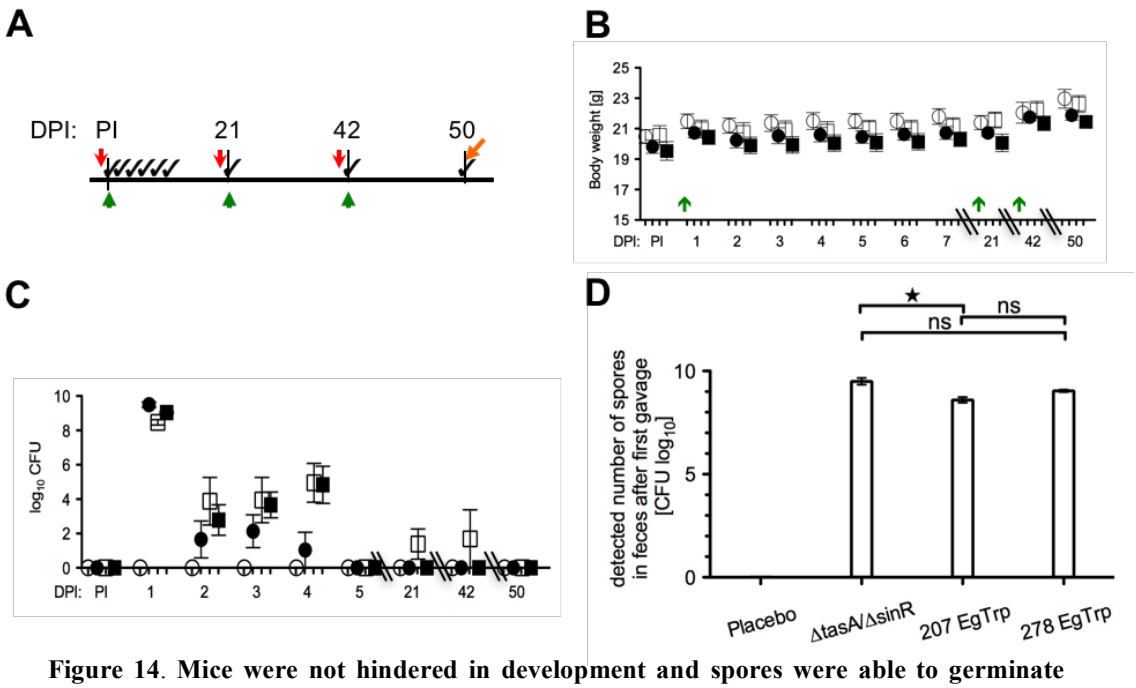


Figure 14. Mice were not hindered in development and spores were able to germinate after the passage through mice. (A) Schematic representation of the experimental design. Recombinant *B. subtilis* strains (102-207)EgTrp and (102-278)EgTrp were administered. Six female, nine weeks old Balb/c mice per group received by oral gavage (↑) 5 x 10^{10} spores or saline solution (Placebo) on days 1, 21 and 42 of the experiment. Before each oral gavage, blood (↓) and feces (✓) samples were collected from each mouse. On day 50 mice were euthanized (↙), and mesenteric lymph nodes, Peyer's patches, and spleen were harvested for analysis. **(B)** Plot showing the mouse health status. The data corresponding to the group body weight represented as mean ± SEM [g]**. (C)** A number of spores detected in the feces of mice resolved in 24 h. Data points correspond to the number of colonies forming units (CFU) of spores formed on selective media, represented as group mean ± SEM [in CFU / g feces]. **(D)** The total number of spores detected in the feces of mice within the seven days after the first oral gavage. Bars represents the total amount of colony forming units (CFU) as group mean ± SEM. For the plots from (B) and (C), each dot corresponds to the group average by □ for 207EgTrp, ■ for 278EgTrp, ○ for Placebo and • for ΔtasA/ΔsinR. DPI, days post immunization; PI, pre-immune. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

The humoral immune response of mice administrated with recombinant spores carrying (102-207)EgTrp and (102-278)EgTrp was analyzed by indirect ELISA. For this purpose, different kind of antigens, such as biofilm extract from recombinant *B. subtilis* (102-207)EgTrp and Δ*tasA/*Δ*sinR*, purified recombinant proteins H_6-EgTrp, H_6-TasA, and H_6-mCherry as well as homogenized *E. granulosus* protoscoleces were used to coat the plates.

In **Figure 15A**, on days 42 and 50 post-inoculation the level of sIgA against complete biofilm of the (102-207)EgTrp strain raised significantly for the group of mice receiving (102-278)EgTrp spores when compared with pre-immunized conditions within the same group. However, the measured values in the ELISA (**Figure 15A**) of serum of mice of the ΔtasA/ΔsinR group against the biofilm of the (102-207)EgTrp strain were on a similar level as the values for the (102-207)EgTrp group. Therefore, the antigen to which the sIgA bind remains not-elucidated. Further does the signal from two mice of the (102-207)EgTrp group against the biofilm of the ΔtasA/ΔsinR strain (**Figure 15B**) underline that other components than the TasA-(102-207)EgTrp is recognized by the sIgA detected. Further, the level of specific IgA in serum against biofilm of the (102-207)EgTrp strain did not show any significant variations, independently of the spores received by the mice (**Figure 15C**).

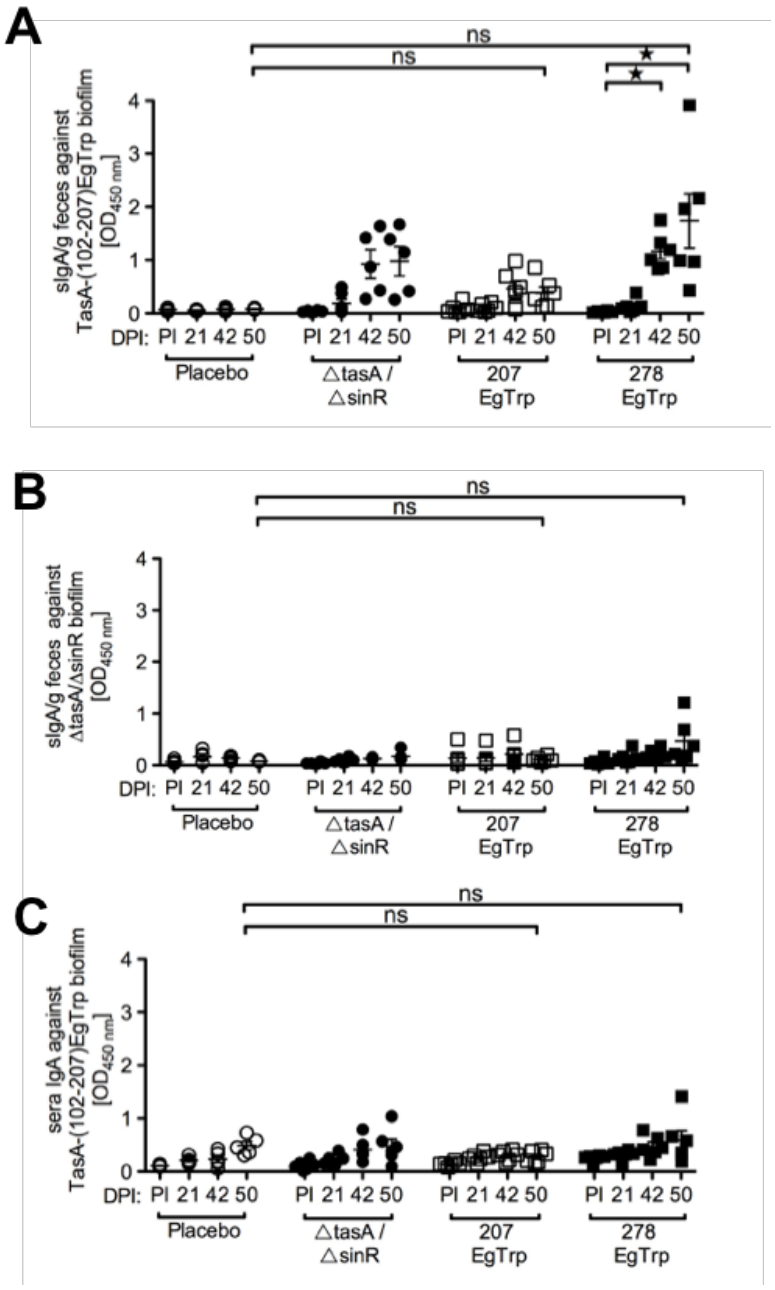


Figure 15. Humoral immune response against components of the *B. subtilis* biofilm.
(A) Plot showing secretory IgA in feces detected by ELISA against homogenate of (102-207)EgTrp biofilm. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. **(B)** Plot showing secretory IgA in feces detected by ELISA against homogenate of ΔtasA/ΔsinR biofilm. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate. **(C)** Plot showing IgA in serum detected by ELISA. Each dot represents the average at $OD_{450\ nm}$ of one mouse in duplicate.
For the plots from (A) to (C): The coated antigens are indicated at the y-axis. The group of mice is shown below the x-axis. Each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, ○ for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

Additional ELISAs were performed for the detection of specific IgA extracted from feces (**Figure 16A**), and serum (**Figure 16B**) directed against purified recombinant proteins H_6-EgTrp, H_6-mCherry and H_6-TasA, biofilm extract from ΔtasA/ΔsinR as well as crude homogenized *E. granulosus* that showed no difference when compared to the placebo

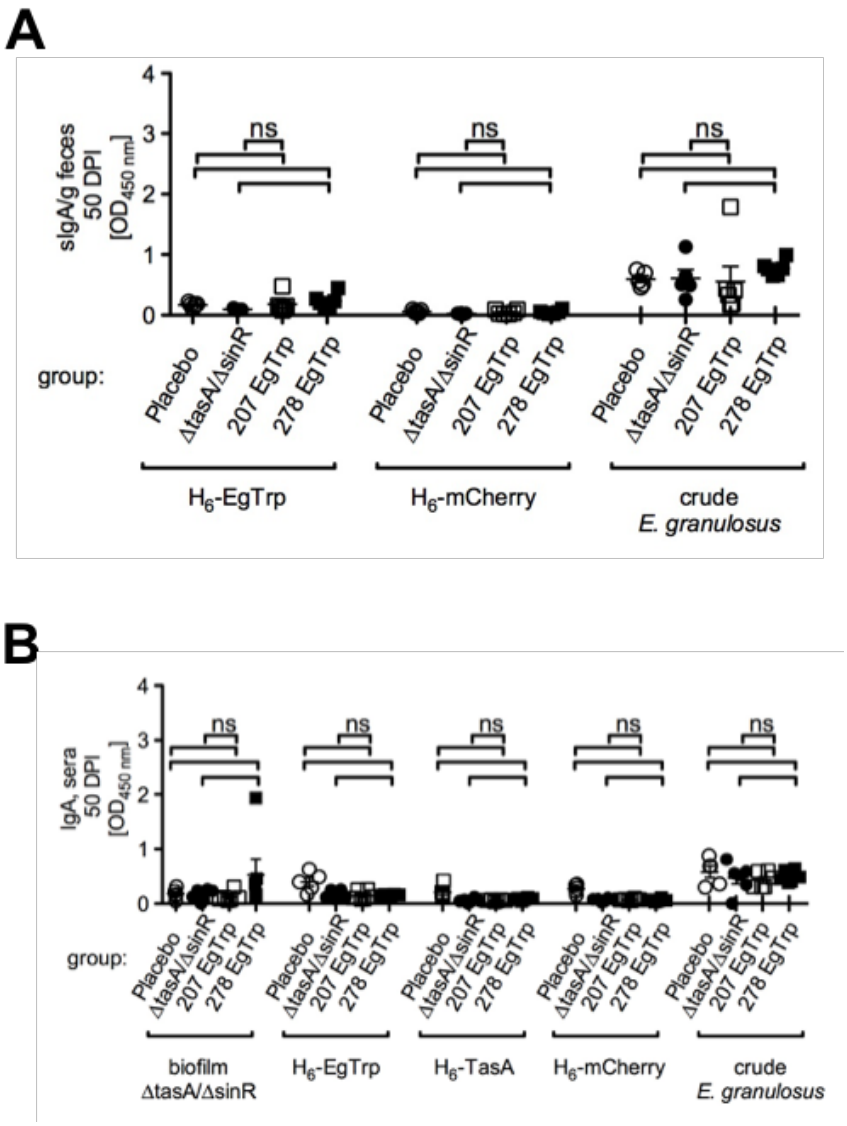


Figure 16. Humoral immune response against purified recombinant components of the *B. subtilis* biofilm.

(A) Plot showing secretory IgA in feces detected by ELISA at 50 dpi. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. **(B)** Plot showing IgA in serum detected by ELISA at 50 dpi. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate.

For the plots (A) and (B): The coated antigens are indicated at the bottom below the x-axis [horizontal text]. The group of mice is shown below the x-axis. Each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

samples. Moreover, the cellular immune response, obtained from T cell proliferation analysis, showed no significant differences when stimulating with purified H_6-EgTrp $CD4^+/CD8^+$ T cells derived from spleen, MLNs and Peyer's patches (**Figure 17**).

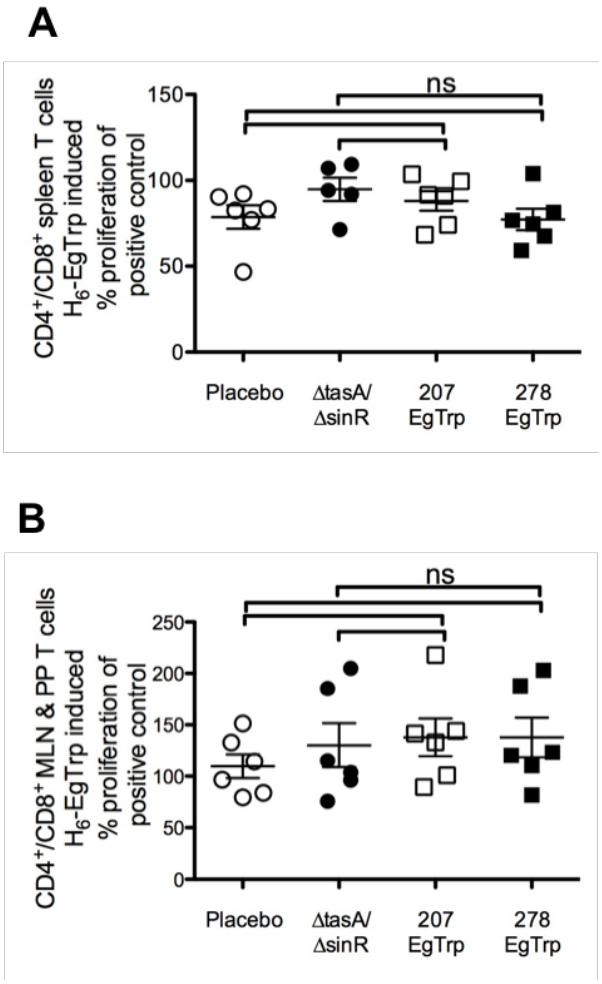


Figure 17. Cellular immune response detected in mice immunized with (102-207)EgTrp and (102-278)EgTrp recombinant spores.
(A) Plot showing proliferating $CD4^+/CD8^+$ cells derived from the spleen after stimulation with purified recombinant H_6-EgTrp. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate. **(B)** Plot showing proliferating $CD4^+/CD8^+$ cells derived from the MLN and Peyer's patches after stimulation with purified recombinant H_6-EgTrp. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate. For the plots from (A) and (B) each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

In summary, within the performed experiments no adverse effects of the recombinant *B. subtilis* have been detected. Furthermore, the oral inoculation with recombinant spores led to a significant increase of sIgA of the (102-207)EgTrp group against components of the biofilm of the (102-207)EgTrp strain. However, it remains not elucidated to which antigenic components in specific as the level of the sIgA from the ΔtasA/ΔsinR group detected against the biofilm of the (102-207)EgTrp strain was on a comparable level as the sIgA detected for (102-207)EgTrp. Further did the analysis of the humoral immune response against purified proteins and crude *E. granulosus* remain negative. The results of the analysis of the cellular immune response showed no significant difference between the groups. Hence, upon these conditions, it was not possible to detect a specific humoral or cellular immune response against recombinant *E. granulosus* antigens.

Oral treatment with antibiotics reduced mice microflora

The gut microflora could inhibit the germination of the administered recombinant *B. subtilis* by a different mechanism, as is the lack of an appropriate niche in the gut or commensalism and thereby, reducing a potential humoral immune response [102]. Thus, to test if an altered microflora would allow a specific immune response against recombinant *E. granulosus* antigens I decided to change the mice original gut microflora by treating the mice with antibiotics. For this purpose, an antibiotic cocktail containing ampicillin, gentamicin, metronidazole and vancomycin was added to the mice drinking water [202]. In a first instance, the effect of the antibiotics treatment on the gut microflora was determined in a pre-test using three mice. In this case, the antibiotic cocktail was provided in the drinking water for six days (**Figure 18A**), in which the mice were weighed, and the feces were harvested on a daily base, respectively. The body weight was monitored as an indicator of the health status of the animals (**Figure 18B**). The weight of mice treated with antibiotics was compared with the untreated mouse. The body weight data shows a loss of weight during days 1 to 3 of the antibiotics treatment, but followed by a stabilization and a regain of weight.

The bacteria content in isolated feces isolated from the mice treated with antibiotics was determined by feces extracts incubated in nutritious, rich agar media as Luria-Bertani (LB), Heart-Brain Infusion (HBI) and Nutrient Broth (**Figure 18 C, D, and E**). The number of colony forming units (CFU) was determined and compared with the feces from the untreated mouse. The data show that CFU of mice treated with antibiotics dropped undetectable levels on day 2 when compared with the untreated mouse. In fact, the number of detected bacteria in feces was not incremented until day 6 of treatment. Same kinds of results were obtained using LB, HBI and NB agar media. Thus, based on these results, the following experiments the mice would receive antibiotics in the drinking water before the oral gavage of recombinant spores.

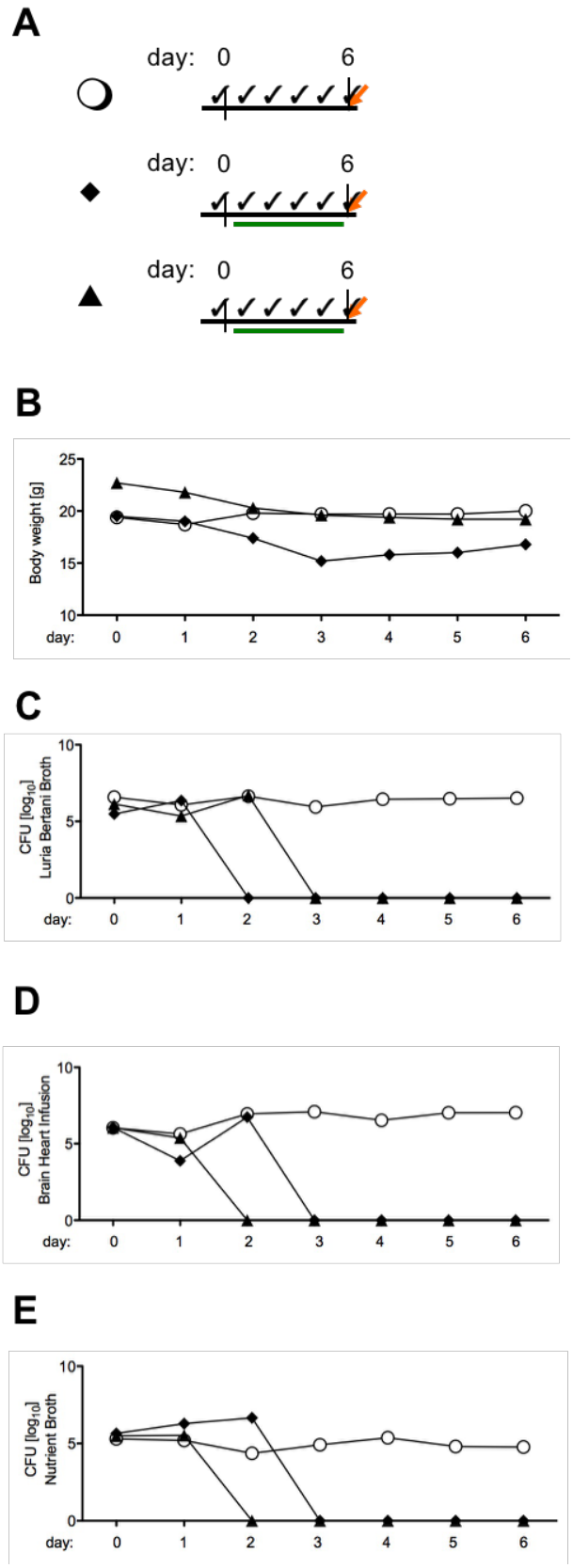


Figure 18: Antibiotics in the drinking water reduce the number of detectable bacteria in the feces. Two mice received antibiotics added to the drinking water (ampicillin, gentamicin, metronidazole, vancomycin), one mouse got normal drinking water. Every 24 h feces (✓) was collected and analyzed for bacteria cultivable on different media. **(A)** Scheme of the experiment for each tested mouse. In green (—) indicated the time of antibiotics in the drinking water. The ticks (✓) represent the daily feces collection, and the red arrow (↙) shows the euthanasia of the mice. **(B)** Health status of the mice. The graph represents the body weight [g] of the mice. Every dot shows one measurement of one mouse. **(C)** Colony forming units of bacteria cultivable on lysogeny broth-agar semi-solid plates. **(D)** Colony forming units of bacteria cultivable on brain heart infusion-agar semi-solid plates. **(E)** Colony forming units of bacteria cultivable on nutrient broth-agar semi-solid plates.

For the graphs B to E each dot represents the $\log_{10}$ of CFU extracted of 24 hours of feces collected and analyzed. The dots represents each mouse by ❍ for the control receiving normal drinking water, ▲ for one mouse receiving antibiotics within the drinking water and the for the other antibiotics receiving mouse.

Evaluation of immune response of mice administrated with recombinant *B. subtilis spores* carrying EgTrp after eradication of the gut microbiota.

In this preliminary schedule (**Figure 19A**), the mice were treated for six days with the antibiotic cocktail in the drinking water. Then, mice receive three doses of $5x10^{10}$ CFU of spores on days 1, 21 and 42. The mice were organized in the following groups: i) placebo (saline solution), ii) control spores ΔtasA/ΔsinR iii) recombinant spores of the (102-207)EgTrp strain and iv) recombinant spores of the (102-278)EgTrp strain. Each group was composed of six female Balb-c mice, six weeks old. The feces and serum were collected to study the humoral immune response and to monitor spores shed in feces. On day 50 post-immunization, mice from all groups were euthanized for the analysis of cellular immune responses from isolated T cells from spleen, mesenteric lymph nodes, and Peyer's patches. The mice body weight was measured daily during the antibiotic treatment and one week after the first immunization to evaluate if the concomitant administration of the antibiotic cocktail and the recombinant spores would have an effect on the mice health. As depicted in **Figure 19B**, no differences in the body weight were observed between mice treated with spores and the control group. However, the treatment with antibiotics had to be interrupted on the fifth day of therapy because the body weight dropped in average more than 20% from the initial body weight in all groups. Hence, mice were provided for one night of drinking water without antibiotics to allow their rehydration. This treatment permits a normalization of the body weight in the next morning and hence the treatment with antibiotics was continued for another two days.

The feces samples were collected from individual animals to determine if the antibiotic treatment disabled the recombinant spores to germinate after the passage through the GIT and to determine the passage time of the recombinant spores. The spores administered were detected in the collected feces within the first four days' post-inoculation for all the animals (**Figure 19C**). In the feces collected on day 21, no spores were detected. Interestingly, when checking the spores shed in the feces from the day 42 (after 21 days from previous oral gavage), one mouse of the (102-207)EgTrp group was shedding 6.41 x 10^5 spores within one day of feces collection. Moreover, in day 50 all animals that have received recombinant spores showed to shed recombinant spores into its feces. Furthermore, the total number of shed spores in feces (**Figure 19D**) after the first oral gavage was determined as 0.2% (ΔtasA/ΔsinR), 31.7% (102-207EgTrp) and 11.3% (102-278EgTrp) from the oral administered dose of spores.

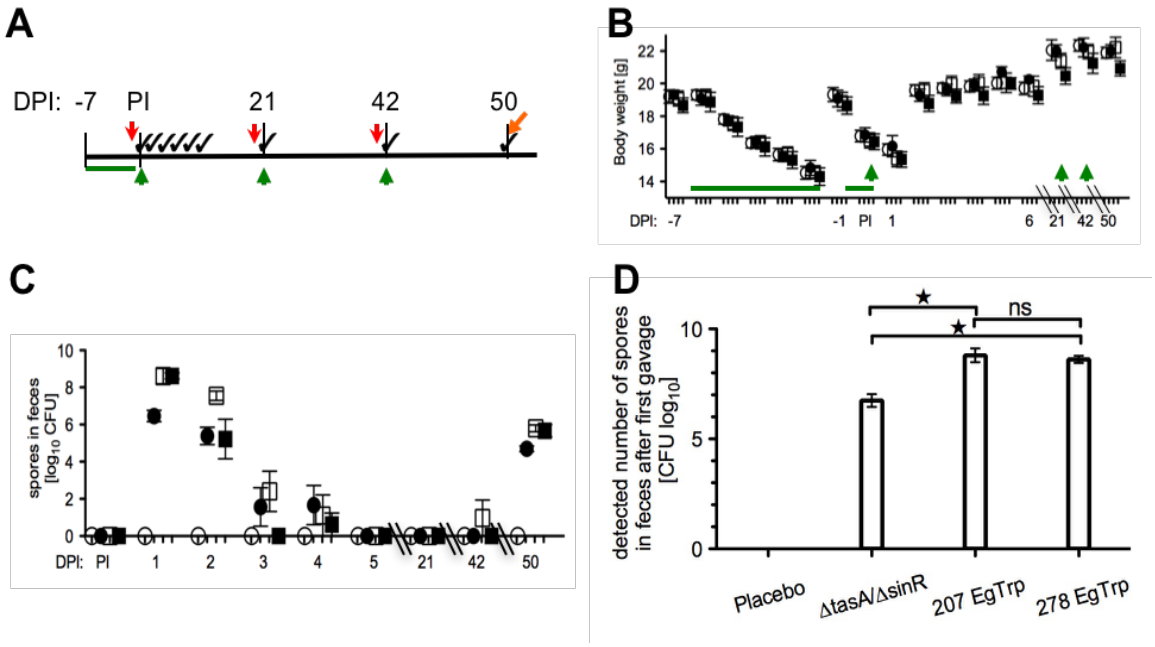


Figure 19: Antibiotics administration lowered the body weight and did not hinder recombinant spores to be able to germinate after the passage through the gastrointestinal tract.

(A) Schematic representation of the spores administration schedule. Recombinant *B. subtilis* strains (102-207)EgTrp and (102-278)EgTrp were administered. Six female, nine weeks old Balb/c mice per group received by oral gavage (🡅) 5 x 10^{10} spores or saline solution (Placebo) on days 1, 21 and 42 of the experiment. The mice received antibiotics in the drinking water before the first oral gavage (___). Before each oral gavage, blood (🡇) and feces (✓) samples were collected from each mouse. On day 50 mice were euthanized (🡇), and mesenteric lymph nodes, Peyer's patches, and spleen were harvested for analysis. **(B)** Plot showing the mice body weight. Data corresponding to the group body weight represented as mean ± SEM [g]. **(C)** Detection of spore amounts in mice feces at 24 h. Data points correspond to the number of colony forming units (CFU) of spores formed on selective media, represented as group mean ± SEM [in CFU / g feces]. **(D)** Total number of spores detected in the feces of mice within seven days after the first oral gavage. Bars represents the total amount of colony forming units (CFU) as group mean ± SEM.

For the plots from (B), and (C) each dot corresponds to the group average by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization; PI, pre-immune. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

To determine the specific humoral immune response against the antigenic peptide from *E. granulosus*, sIgA extracted from feces, and IgA from serum were analyzed by indirect ELISA. As depicted in **Figure 20A**, mice inoculated with recombinant spores of both strains, (102-207)EgTrp and (102-278)Egtrp, were able to respond specifically against homogenized biofilm. Mice of the (102-207)EgTrp group showed an increased signal on days 42 and 50 post-immunization represented by specific sIgA isolated from feces. The mice group immunized with spores carrying (102-278)EgTrp showed on 42 days post-

inoculation a significant increase of sIgA against the homogenized (102-207)EgTrp biofilm compared to the preimmune sample. Meanwhile, the control groups (placebo and ΔtasA/ΔsinR) were unable to generate such a response against the biofilm extract. However, the difference between the response on day 50 of the EgTrp spores receiving groups was not significantly different from the signal detected testing the day 50 serum of the placebo group. Importantly, no signal was obtained from biofilm extract from *B. subtilis* ΔtasA/ΔsinR (**Figure 20B**), pointing into the direction that mice responded specifically against the TasA-EgTrp fusion protein. When analyzing the specific antibody response in serum from the gavaged mice, in particular for IgA (**Figure 20C**), it was possible to observe a significant increase of IgA against (102-207)EgTrp biofilm for all three groups receiving recombinant *B. subtilis* spores. The results indicate a non-specific immune response against other components of the biofilm than TasA-(102-207)EgTrp.

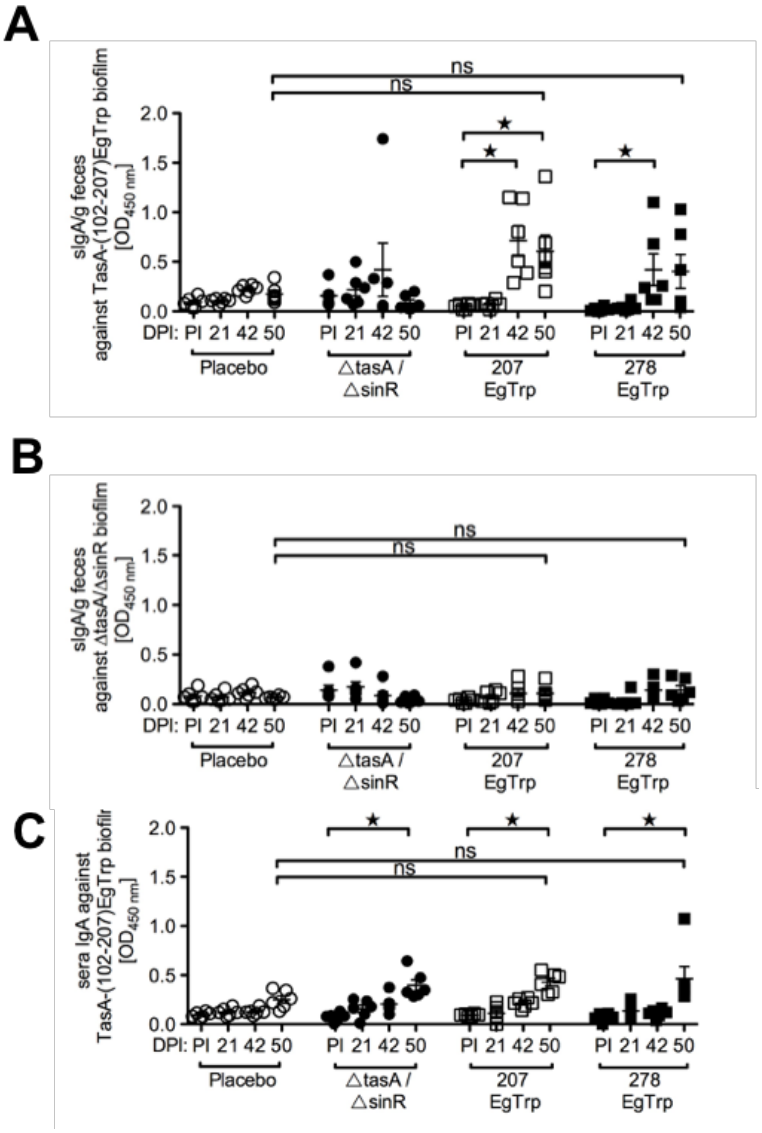


Figure 20: Specific secretory IgA of mice immunized with (102-207)EgTrp and (102-278)EgTrp recombinant spores detected after treatment of the mice with antibiotics before the first oral gavage.

(A) Plot showing secretory IgA in feces detected by ELISA coated with homogenized biofilm expressing TasA-(102-207)EgTrp. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. **(B)** Plot showing secretory IgA in feces detected by indirect ELISA coated with 72h homogenized biofilm from B. subtilis ΔtasA/ΔsinR strain. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. **(C)** Plot showing serum IgA detected by ELISA coated with 72h homogenized biofilm from B. subtilis TasA-(102-207)EgTrp strain. Each dot represents the mean value at $OD_{450\ nm}$ for one mouse in duplicate.

For the plots from (A) to (C) each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, ○ for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization; PI, pre-immune. Error bars show the standard error of the means of each group. ★, p-value < 0.05; ns, not significant; one-way ANOVA with Tukey's multiple comparison tests.

Interestingly, sIgA isolated from feces (**Figure 21A**) of mice gavaged with recombinant spores carrying EgTrp antigenic peptides were able to recognize (ns) wild-type biofilm extract, possibly pointing to recognize TasA in the biofilm. However, no signal was detected from other substrates as recombinant proteins for H_6-EgTrp and H_6-mCherry and also crude *E. granulosus*. Thus, no sera IgA were detected against homogenate of the biofilm of the ΔtasA/ΔsinR strain, and also no significant increase in serum IgA against homogenized biofilm of the wt strain was detected (**Figure 21B**). The sera IgA against H_6-EgTrp, H_6-mCherry, and crude *E. granulosus* did not change detectable.

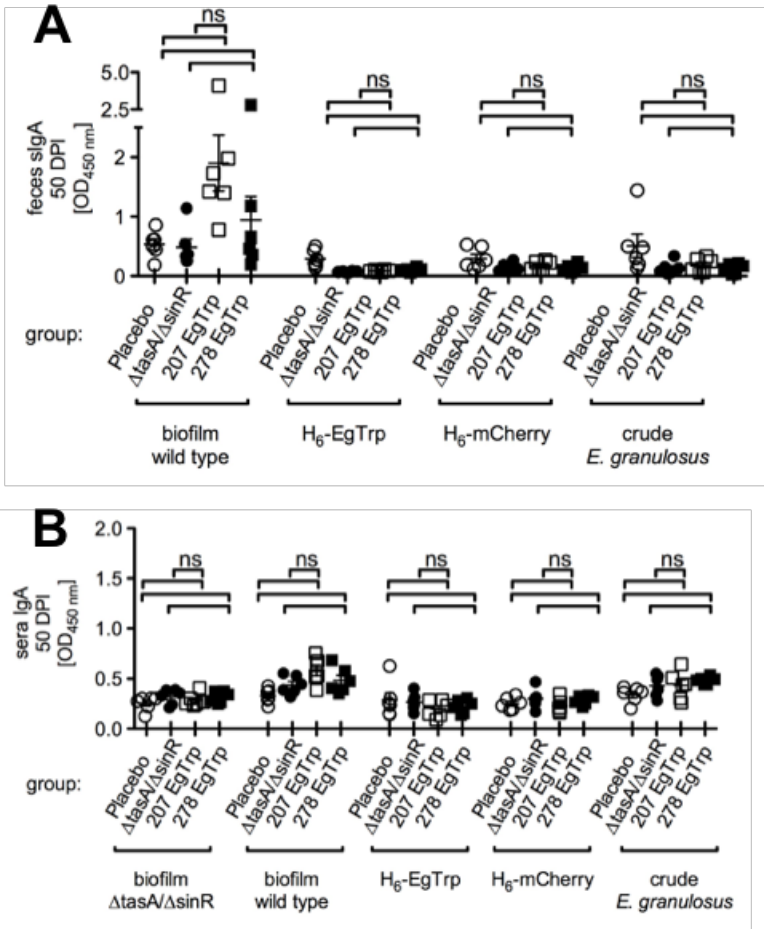


Figure 21: 50 DPI Intestinal humoral immune response of mice immunized with (102-207)EgTrp and (102-278)EgTrp recombinant spores detected against homogenate of wt *B. subtilis* biofilm

(A) ELISA plot is showing secretory IgA in feces at 50 dpi. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. (**B**) Plot showing serum IgA at 50 doi detected by ELISA. Each dot represents the mean at $OD_{450\ nm}$ for one mouse in duplicate.

For the plots (A) and (B); the coated antigens are indicated at the bottom [horizontal text]. The group of mice is shown below the x-axis. Each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, ○ for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

The cellular immune response from isolated $CD4^+/CD8^+$ T cells from spleen, MLN and Peyer's patches was determined by dilution of incorporated CFSE in proliferative T cells. Unfortunately, as depicted in **Figure 22**, no significant specific cellular immune response was observed for all the groups tested when stimulating with purified recombinant H_6-EgTrp or crude homogenized *E. granulosus* antigens.

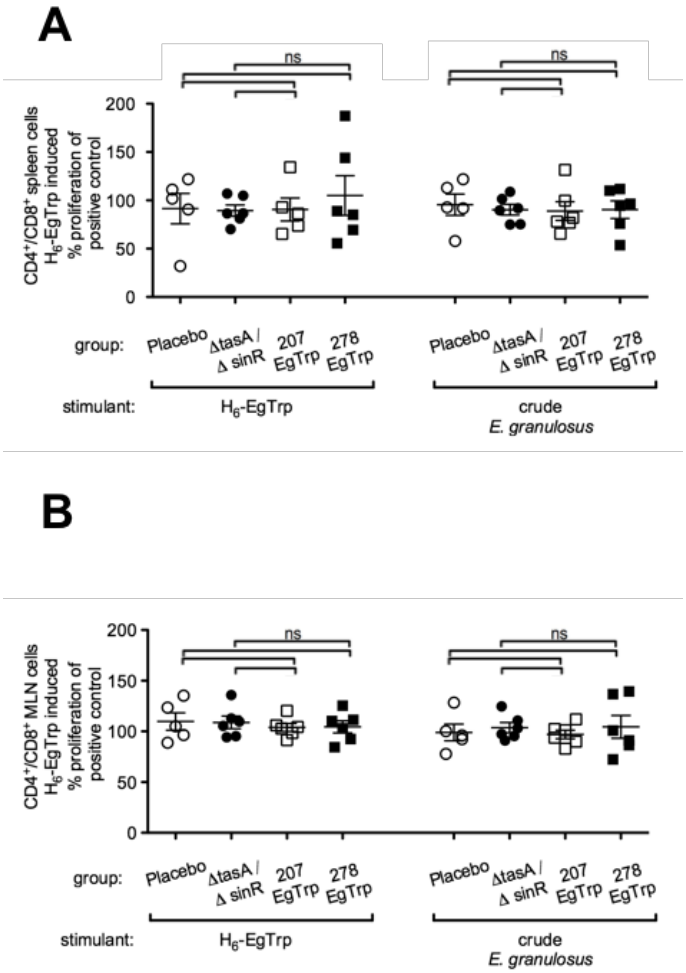


Figure 22: Cellular immune response remains unaltered independent of the group of the mice.

(A) Plot showing proliferating $CD4^{+/}CD8^{+}$ cells derived from the spleen after stimulation with H_6-EgTrp or homogenized crude *E. granulosus* indicated below x-axis [horizontal text]. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate.

(B) Plot showing proliferating $CD4^{+/}CD8^{+}$ cells derived from the MLN and Peyer's patches after stimulation with H_6-EgTrp or homogenized crude *E. granulosus* indicated below x-axis [horizontal text]. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate.

For the plots from (A) to (B), each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. Error bars show the standard error of the arithmetic mean of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

Thus, a specific humoral response of sIgA against homogenized (102-207)EgTrp biofilm for both groups of EgTrp spore receiving mice was observed. Based on the obtained results it was concluded that a local intestinal humoral immune response, particularly

with the (102-207)EgTrp group could be detected when treating the mice with antibiotics previous to the first oral gavage with recombinant spores. However, it remains unenlightened against which components of the *B. subtilis* biofilm the immune response was directed.

Results of the mouse experiment of mice treated previously to each oral gavage with antibiotics

With the previous experiments, I had shown that modeling the intestinal microflora before the first inoculation improved the detected local humoral immune response against the EgTrp antigenic peptides. Due to the role of the antibiotics in eradicating the gut microflora, I questioned the effect that it would have to the immune response when mice were treated with antibiotics before each immunization boost with the recombinant spores. For this purpose, an experiment including the following schedule was performed (**Figure 23A**).

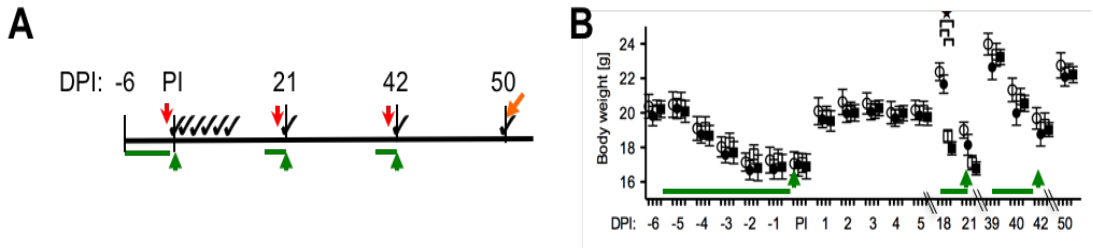


Figure 23: Mouse experiment with animals receiving antibiotics before each oral gavage of recombinant spores expressing EgTrp in the ECM of biofilms.
(A) Schematic representation of the inoculation schedule. Recombinant *B. subtilis* strains (102-207)EgTrp and (102-278)EgTrp were administered. Six female, six weeks old Balb/c mice per group received through oral gavage (↑) 5 x 10^{10} spores or saline solution (Placebo) on days 1, 21 and 42 of the experiment. The mice received antibiotics in the drinking water before each oral gavage (-). Before each oral gavage, blood (↓) and feces (✓) samples were collected from each mouse. On day 50 mice were euthanized (↙), and mesenteric lymph nodes, Peyer's patches, and spleen were harvested for analysis. **(B)** Plot showing mouse body weight. Data corresponding to the group body weight represented as mean ± SEM [g]. Each dot corresponds to the group average by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization; PI, pre-immune. ↑, oral gavage. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05.

The mice were grouped into four groups of 6 mice each: i) placebo (saline solution), ii) control spores ΔtasA/ΔsinR, iii) recombinant spores of the (102-207)EgTrp strain and iv) recombinant spores of the (102-278)EgTrp strain. Mice were immunized by oral gavage on days 1, 21 and 42 of the schedule. Besides, the mice received the antibiotic cocktail for

six days prior the first oral gavage and three days previous the 2nd and third immunization (days 21 and 42). Feces and blood samples were collected as indicated in the immunization schedule. At day 50, mice were euthanized for collection of spleen, MLN and Peyer's patches. As expected during each treatment with antibiotics the mice had lost weight but always less than 20% of their initial weight (**Figure 23B**). Additionally, they recovered within a short time once the antibiotic treatment was interrupted.

Indirect ELISA measured the local immune response from sIgA isolated from feces in plates coated with biofilm from *B. subtilis* (102-207)EgTrp strain (**Figure 24A**). The mice of the (102-278)EgTrp group showed to have a significant higher sIgA level on day 50 when compared to the Placebo mice. However, the level of sIgA was similar to the level of sIgA that ΔtasA/ΔsinR receiving mice showed on day 42. Further showed the mice of all the groups gavaged with spores a higher level of sIgA at day 42 when compared to the placebo when coating with biofilm extract from *B. subtilis* ΔtasA/ΔsinR or wild type (**Figures 24B and 25A**). Furthermore, the serum IgA (**Figures 24 C and 25 B**), showed a specific response of both EgTrp spores receiving groups against biofilm extracts of *B. subtilis* (102-207)EgTrp but not against ΔtasA/ΔsinR strains and to purified recombinant proteins H_6-EgTrp, H_6-TasA and H_6-mCherry. However, it remains not-elucidated to what component of the *B. subtilis* biofilm the mice developed an immune response detected by high sIgA and IgA levels.

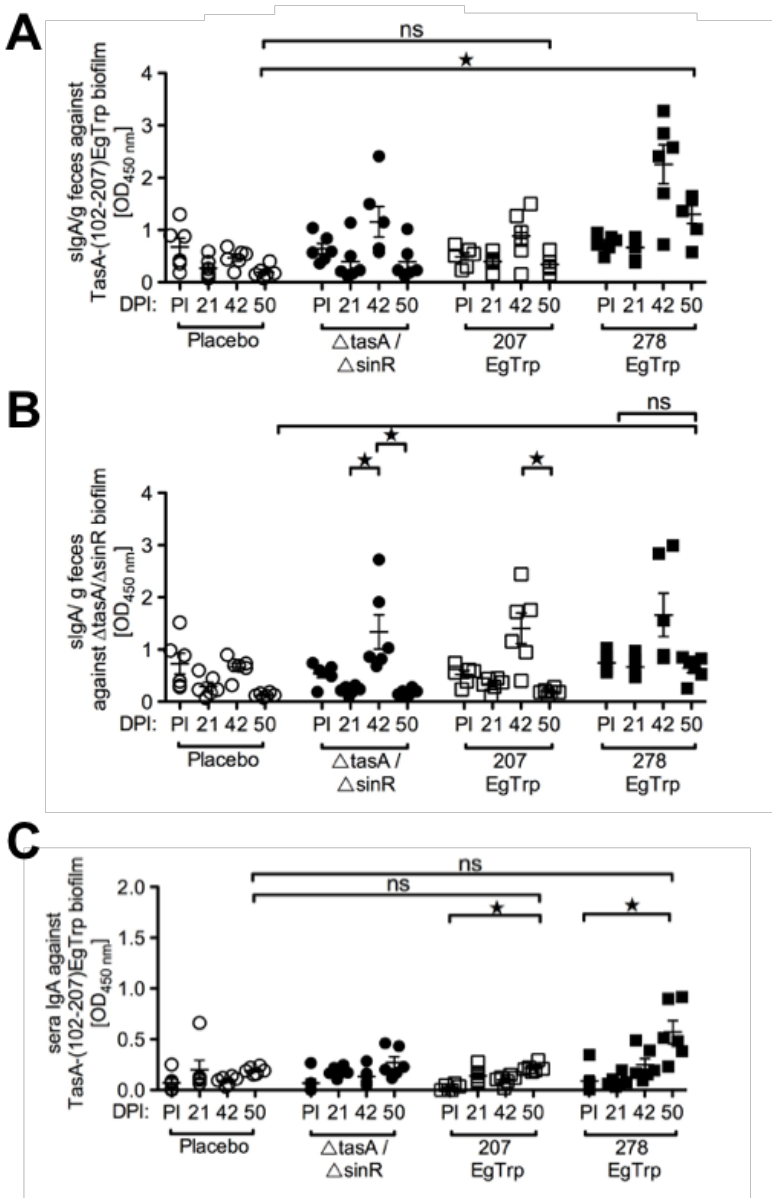


Figure 24: Specific secretory IgA and serum IgA humoral immune response of mice gavaged with (102-278)EgTrp recombinant spores detected after treatment of the mice with antibiotics before each oral gavage.

(A) Plot showing secretory IgA in feces detected by ELISA coated with homogenized biofilm expressing TasA-(102-207)EgTrp. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. **(B)** Plot showing secretory IgA in feces detected by ELISA coated with 72 h homogenized biofilm from B. subtilis ΔtasA/ΔsinR strain. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate and normalized by the analyzed feces weight. **(C)** Plot showing serum IgA detected by ELISA coated with 72h homogenized biofilm B. subtilis TasA-(102-207)EgTrp strain. Each dot represents the mean $OD_{450\ nm}$ of one mouse in duplicate.

For the plots from A to C each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. DPI, days post immunization; PI, pre-immune. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

The cellular immune response of T cells derived from the spleen (**Figure 26**) and pooled MLN and Peyer's patches (**Figure 27**) was determined by a proliferation assays using for stimulation purified H_6-EgTrp, H_6-TasA and homogenized crude *E. granulosus* antigens. The analysis of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ T cells did not show any significant differences between the analyzed groups.

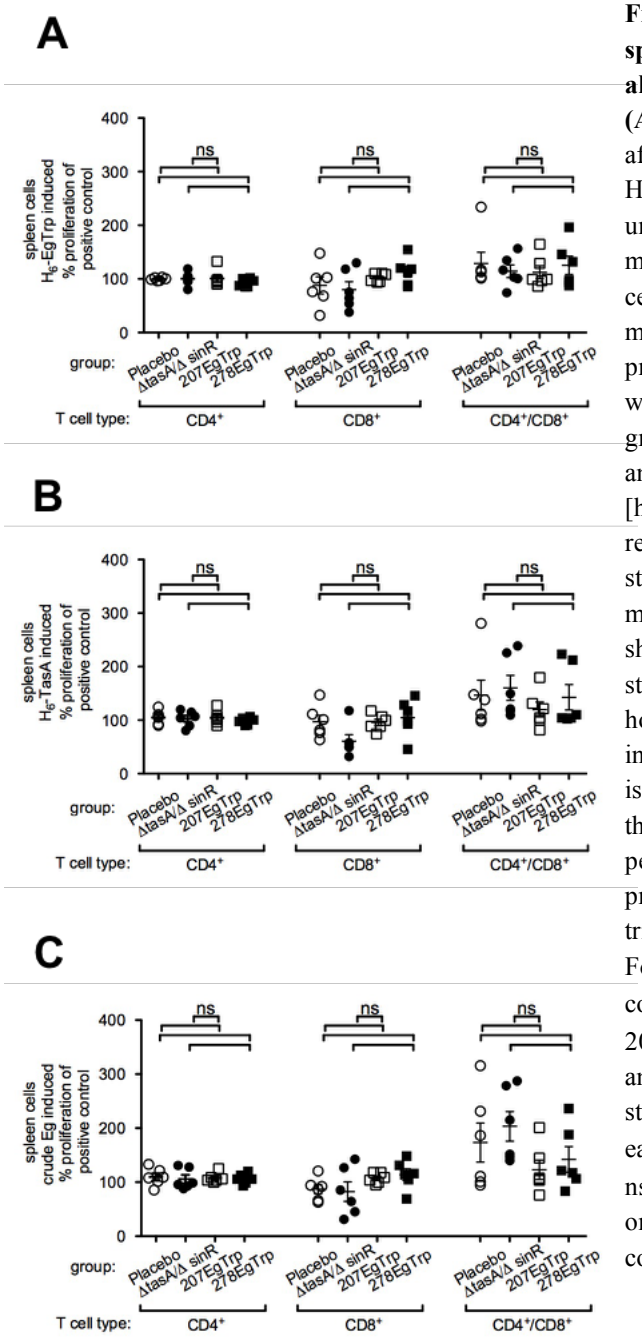


Figure 26: Cellular immune response of spleen-derived cells remains unaltered for all groups.

(A) Plot showing proliferating spleen cells after stimulation with purified recombinant H_6-EgTrp. The group of mice is indicated under the x-axis. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate. **(B)** Plot showing proliferating spleen cells after stimulation with purified recombinant H_6-TasA. The group of mice is indicated below the x-axis, and the cell type is indicated at the bottom [horizontal text] of the plot. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate. **(C)** Plot showing proliferating spleen cells after stimulation with crude *E. granulosus* homogenized. The group of mice is indicated below the x-axis, and the cell type is indicated at the bottom [horizontal text] of the plot. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate.

For the plots from A to C each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, ○ for Placebo and ● for ΔtasA/ΔsinR. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

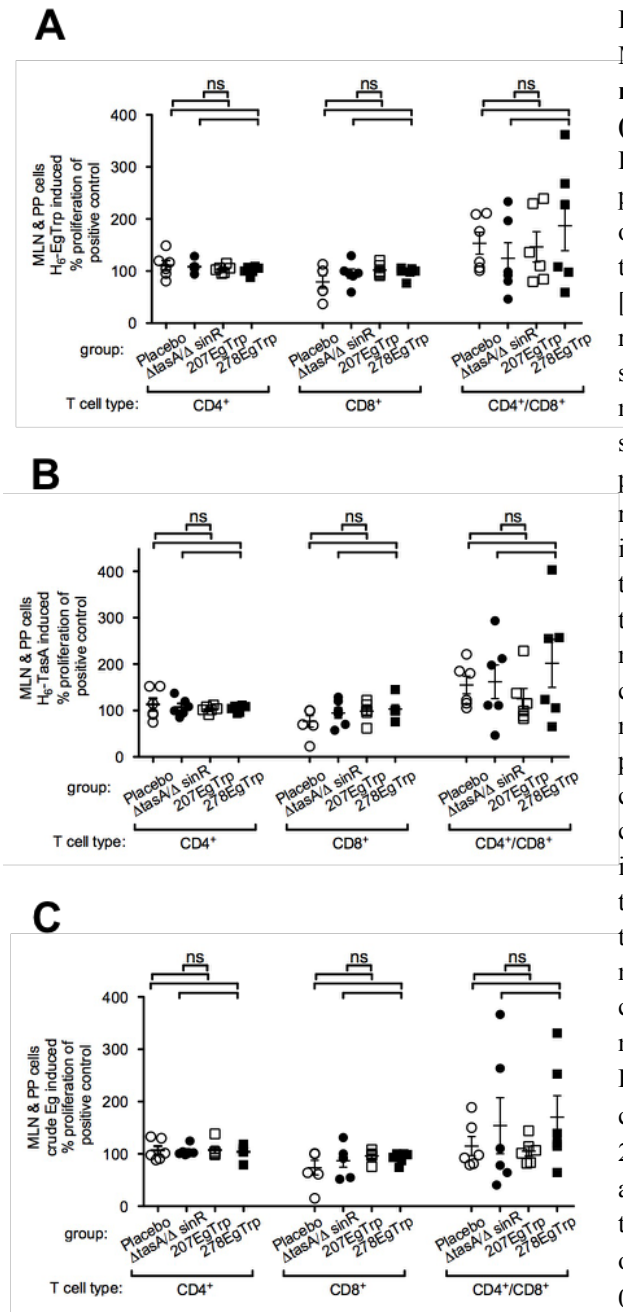


Figure 27: Cellular immune response of MLN and Peyer's patches derived cells remains unaltered for all groups.
(A) Plot showing proliferating MLN and Peyer's patches cells after stimulation with purified recombinant H_6-EgTrp. The group of mice is indicated below the x-axis, and the cell type is indicated at the bottom [horizontal text| of the plot. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate. **(B)** Plot showing proliferating MLN and Peyer's patches cells after stimulation with purified recombinant H_6-TasA. The group of mice is indicated below the x-axis, and the cell type is indicated at the bottom [horizontal text] of the plot. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate. **(C)** Plot showing proliferating MLN and Peyer's patches cells after stimulation with homogenized crude *E. granulosus*. The group of mice is indicated below the x-axis, and the cell type is indicated at the bottom [horizontal text] of the plot. Each dot represents the mean percentage of maximal stimulated cellular proliferation of one mouse measured in triplicate.
For the plots from A to C each dot corresponds to one mouse by □ for 207EgTrp, ■ for 278EgTrp, O for Placebo and ● for ΔtasA/ΔsinR. Error bars show the standard error of the arithmetic means of each group. ★ indicates a p-value < 0.05, ns indicates a not significant difference; one-way ANOVA with Tukey's multiple comparison tests.

Thereby by treating the mice with antibiotics previous to each oral gavage seemed not to increase the humoral immune response. The obtained data suggest that for an enhancement of the humoral immune response it is sufficient to provide a unique treatment with antibiotics at the beginning of the enteric immunization. Furthermore, after

repeated antibiotics treatment its favor the antigenicity of the (102-278)EgTrp spores over the (102-207) spores.

Evaluation of the immune response in mice orally administrated with recombinant *B. subtilis* spores carrying EgA31 antigenic peptide.

Parallel to the testing of *E. granulosus* tropomyosin (EgTrp) antigens by enteric immunization: I tested if the recombinant *B. subtilis* expressing (370-583)EgA31, would be able to promote a specific local humoral immune response. For this purpose, two mouse experiments were performed. In the first experiment using TasA-Paramyosin (370-583)EgA31 a schedule was organized that includes an antibiotics treatment before the first and the third oral gavage of recombinant spores. In the second experiment, the schedule includes an antibiotics treatment before each of the three oral gavages. All other parameters were unaltered compared to the previously described experiments using recombinant *B. subtilis* strains expressing EgTrp (**Data not shown**).

The results showed that the mice receiving spores of the *B. subtilis* (370-583)EgA31 strain had a similar behavior as EgTrp strains with a loss of body weight accordingly to the antibiotics treatment but not due to the oral administration of *B. subtilis* spores. The analysis of the local immune response by indirect ELISA of sIgA isolated from the feces revealed no significant difference in the response among the different immunized groups in plates coated with *B. subtilis* biofilm extract from (370-583)EgA31, ΔtasA/ΔsinR or wild-type strains. Also, the systemic humoral response, denoted by serum IgA showed no significant difference between the immunized groups independently of the tested antigens. Also, no difference was observed when stimulating T cells derived from the spleen, MLN and Peyer's patches with purified recombinant H_6-EgA31 or crude *E. granulosus* homogenate. These observations were independent of the experimental setup, therefore independent of giving before the first and third oral gavage or before each oral gavage antibiotics to the mice.

In summary, the spores carrying (370-583)EgA31 were not able to induce a detectable local humoral response even if mice were treated with antibiotics previous to each immunization. The results suggest that these particular recombinant spores were non-immunogenic or induced tolerance.

Use of recombinant *B. subtilis* expressing *E. granulosus* antigens for enteric immunization of dogs

Canidae, like dogs, are the definitive host for the *E. granulosus* tapeworm [203]. Hence, it is of priority to determine if recombinant *B. subtilis* spores carrying *E. granulosus* antigens can induce a local humoral or cellular immune response in orally immunized dogs. For this purpose, an experimental trial was performed in dogs to determine the local and systemic humoral as well as a cellular immune response in dogs orally administered with spores of the recombinant *B. subtilis* strains expressing the *E. granulosus* antigenic peptides, EgA31 and EgTrp.

Schedule of the dog experiment

The tentative schedule was based on the oral administration of recombinant *B. subtilis* spores carrying (102-207)EgTrp, (102-278)EgTrp and (370-583)EgA31 antigenic peptides. Thus, eight animals included in the experiment were separated into four groups of two dogs each. The groups were the following: i) placebo (saline solution), ii) EgTrp (receiving (102-207)EgTrp and (102-278)EgTrp spores), iii) EgA31 (receiving (370-583)EgA31 spores), iv) Mixture (receiving (102-207)EgTrp and (370-583)EgA31 spores). As depicted in the experiment schedule (**Figure 28A)**, the dogs were orally administered with a total $5x10^{10}$ CFU / dose of spores for three times on days 1, 21 and 42. Additionally, the animals were weighed (**Figure 28B**) and examined by a veterinarian before every treatment to ensure their health. Blood samples were collected weekly to determine the humoral and the cellular immune response. Furthermore, on day 60 the dogs were euthanized for the collection of spleen, MLN and Peyer's patches for the analysis of the cellular immune response. Samples from duodenum, jejunum, ileum, caecum and colon were collected for detection of recombinant *B. subtilis* as well as inflammatory cells by histological analysis.

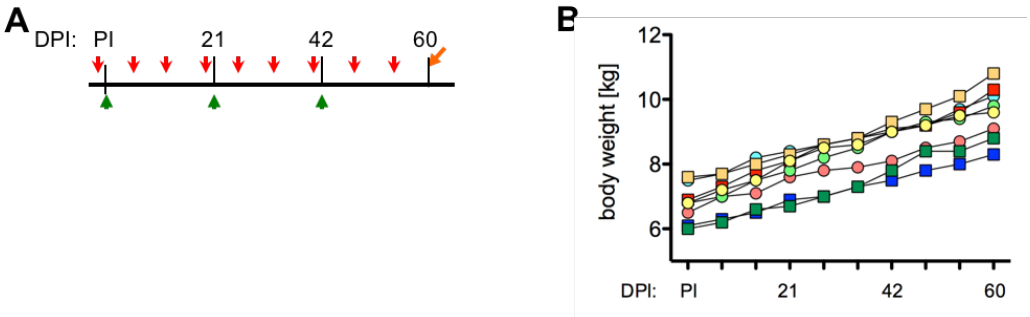


Figure 28: Healthy development of the dogs included in the oral gavage experiment
(A) Schematic representation of the dog immunization schedule with recombinant *B. subtilis* spores (102-207)EgTrp and (102-278)EgTrp or/and (370-583)EgA31 strains. Two beagle dogs per group received by oral gavage (↑) 5 x 10^{10} spores or saline solution (Placebo) on days 1, 21 and 42 of the experiment. Before each oral gavage, blood (↓) samples were collected. On day 60 the dogs were euthanized (↙), and mesenteric lymph nodes, Peyer's patches, spleen and intestinal samples were collected for analysis. **(B)** Plot showing the dog body weight [kg]. Each dot corresponds to one dog bright blue (circle) and dark blue (square) for EgTrp; bright yellow (circle) and dark yellow (square) for EgA31; bright green (circle) and dark green (square) for spores mixture and bright red (circle) and dark red (square) for the placebo.

Humoral immune response of the dogs

The humoral immune response (IgA and IgG) against the recombinant spores was analyzed by indirect ELISA using plates coated with biofilm extracts from *B. subtilis* (102-207)EgTrp or (370-583)EgA31 strains, and purified recombinant proteins H_6-EgTrp, H_6-EgA31, H_6-TasA or H_6-mCherry, respectively. The results are presented as bar charts showing the delta OD, as the pre-immune value was subtracted from the measured OD to determine the specific humoral immune response developed during the experiment. The immunological profiles obtained by the ELISAs of the purified recombinant proteins H_6-EgTrp, H_6-EgA31 and H_6-TasA were compared to the immune response profile of a complete exogenous antigen as is H_6-mCherry. Thereby, the animals that responded positively for H_6-mCherry with an immune profile similar to the other tested recombinant antigens (H_6-EgTrp, H_6-EgA31, H_6-TasA) were considered as not showing specific IgG and or IgA against the tested recombinant antigens. Further, were indirect ELISAs performed against homogenate of complete biofilms from the *B. subtilis* (102-207)EgTrp strain and compared to the immune profile obtained when tested against homogenate of complete biofilms from *B. subtilis* (370-583)EgA31 strain, and vice versa. Thereby, animals that showed a response against the homogenate of biofilm of both strains were considered as responding to more antigens in the biofilm than the specific expressed (102-207)EgTrp and (370-583)EgA31, respectively.

In a first attempt, the serum samples were tested against the purified recombinant protein H_6-mCherry to control for the potentially recognized *E. coli* cell debris from the purification process **(Figure 29)**. In a second attempt, the serum samples were tested against the purified recombinant proteins H_6-EgTrp, H_6-EgA31, and H_6-TasA (**Figure 30**). The bar charts are showing the delta OD of the measured value subtracted the value measured for pre-immune samples.

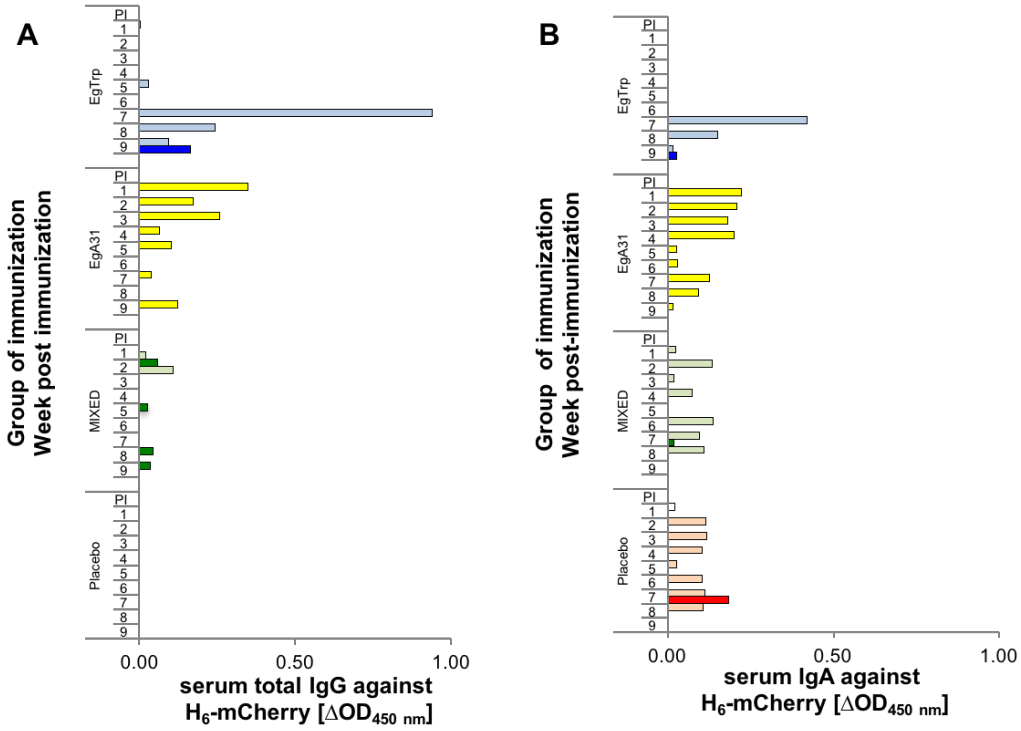


Figure 29: ELISA analysis of the detectable immunoglobulins against the purified recombinant H_6-mCherry

Bar charts are showing the humoral immune response (total IgG and IgA) detected by indirect ELISA. The results were calculated by subtraction of the individual PI level of each dog. In blue (bright and dark) the EgTrp group, in yellow (bright and dark) the EgA31 group, in green (bright and dark) the Mixed group and in red (bright and dark) the Placebo group**. (A)** serum total IgG specific against H_6-mCherry. **(B)** serum IgA specific against H_6-mCherry.

The delta OD presented in **Figure 29 A and B** for one of the dogs receiving EgTrp spores (bright blue bars) increased from seven weeks post-immunization (IgG and IgA). However, one of the dogs receiving (370-583)EgA31 spores (dark yellow bars) showed a decreasing IgG and IgA signal against H_6-mCherry in the ELISA. Further, one of the mixed group dog (dark green bars) show low IgG reaction (< 0.1 ΔOD_{450}), and the other

dog of the mixed group (bright green bars) and the control dog of the Placebo group (bright red bars) showed reactive IgA against H_6-mCherry.

In a next attempt, the detectable IgG and IgA in the blood serum samples of the dogs were analyzed in an ELISA against recombinant H_6-EgTrp. The one dog EgTrp inoculated (dark blue bars) and the dog inoculated with mixed spores (dark green bars) showed increasing IgG and IgA response against purified H_6-EgTrp in the ELISA (**Figure 30 A and B**). Further, one of the dogs receiving EgTrp spores (bright blue bars) show a response for IgG at 3, 7 and 9 weeks post-immunization and for IgA at weeks 7, 8 and 9. But, one of the (370-583)EgA31 spores receiving dogs (dark yellow bars) showed a decrease of IgG and IgA against purified H_6-EgTrp.

When analyzing the ELISA against purified H_6-EgA31 (**Figure 30 C and D**), it was observed that EgTrp spores receiving dogs (bright and dark blue bars) had an increasing response as well as one from mixed spores group (dark green bars) that also had shown previously low response against purified H_6-EgA31. However, (370-583)EgA31 spores receiving dog (dark yellow bars) showed a decreased level of IgG and IgA against H_6-EgA31.

Next, the dog's serum samples were tested against H_6-TasA (**Figure 30 E and F**). A sharp increase of the signal for IgG was detected for the dog receiving spores of the EgTrp strains represented by dark blue bars. Further showed the same dog to have at week nine an increased IgA delta OD against H_6-TasA. Similar to that but only starting from 7 weeks post-immunization and later showed the second EgTrp spore receiving dog (bright blue bars) an increased IgG as well as an IgA delta OD against H_6-TasA. Different from the two (102-207)EgTrp dogs did one of the mixed group dogs (dark green bars) showed a decrease of the signal over time for IgG as well for IgA.

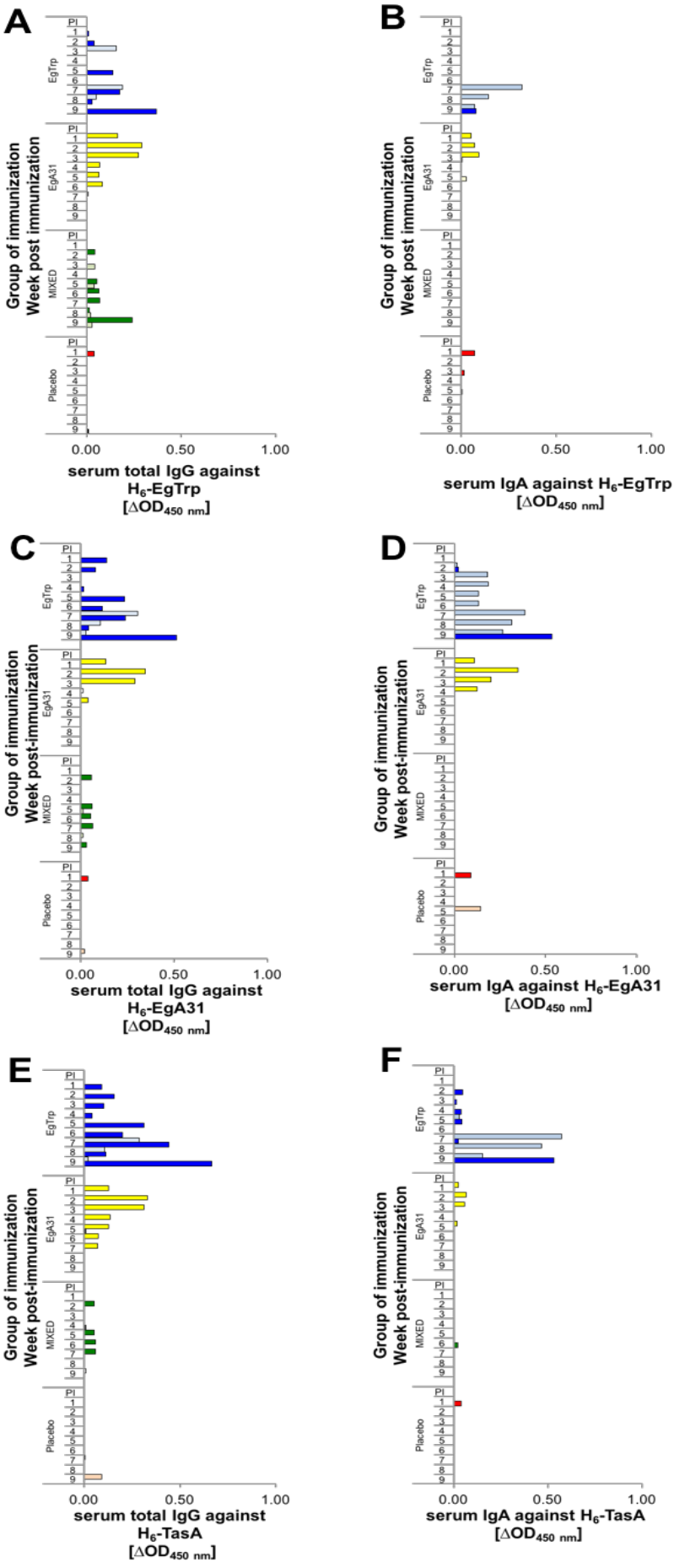


Figure 30: Testing of the humoral response of dogs inoculated with recombinant *B. subtilis* spores against purified recombinant *E. granulosus* proteins.

Bar charts are showing the humoral immune response (total IgG and IgA) detected by indirect ELISA. The results were calculated by subtraction of the individual PI level of each dog. In blue (bright and dark) the EgTrp group, in yellow (bright and dark) the EgA31 group, in green (bright and dark) the Mixed group and in red (bright and dark) the Placebo group.

(A) serum total IgG specific against H_6-EgTrp.

(B) serum IgA specific against H_6-EgTrp.

(C) serum total IgG specific against H_6-EgA31.

(D) serum IgA specific against H_6-EgA31.

(E) serum total IgG specific against H_6-TasA.

(F) serum IgA specific against H_6-TasA

To interpret the results of the ELISA experiment the observed results against purified recombinant H_6-mCherry antigen was compared to the signal profile obtained in the ELISAs against H_6-EgTrp, H_6-EgA31 and H_6-TasA. As the H_6-mCherry was achieved by the same purification process as the other recombinant proteins but was an entirely exogenous antigen for the dogs, the ELISA against H_6-mCherry was expected to show signals from antibodies binding to proteins of *E. coli* remaining from the purification. For one dog receiving spores of the EgTrp strains (dark blue bars) the ELISA analyzing IgG against H_6-EgTrp, H_6-EgA31 and H_6-TasA showed a sharp increase over time of the experiment, whereas the ELISA against H_6-mCherry didn't show a signal apart from a peak on week 9 post-immunization. However, this dog showed a signal in the ELISA for IgA and IgG against H_6-EgA31, which is also an exogenous antigen as H_6-mCherry. Then the dog (dark blue bars) inoculated by (102-207) EgTrp spores was probably not showing a specific response against the recombinant H_6-EgTrp, H_6-EgA31, and H_6-TasA. However, an increase of the signal over time was detected, which could not be differentiated against what component it was directed. Different to that, one dog receiving the mixture of *B. subtilis* spores of the (102-207)EgTrp and the (370-583)EgA31 strain (dark green bars) showed an increase of signal of IgG against H_6-EgTrp, H_6-EgA31 and H_6-TasA detectable by ELISA. The other dogs had shown an ELISA signal profile against H_6-EgTrp, H_6-EgA31 and H_6-TasA comparable to the ELISA signal profile for H_6-mCherry or no signal at all and were therefore counted as not having developed a detectable humoral immune response specific against H_6-EgTrp, H_6-EgA31 and H_6-TasA but probably against debris of *E. coli* lasting from the purification process.

Next, the serum samples were tested first against biofilm from (102-207)EgTrp and second against biofilm of the (370-583)EgA31strain in an ELISA analysis (**figure 31**). The dog receiving spores of the EgTrp strains (dark blue bars) as well as the dog receiving the mixture of spores (dark green bars) showed a positive IgG delta OD from week two post-immunization on against the homogenate from the (102-207)EgTrp biofilm. For the IgA response, the dark green dog showed from seven weeks post-immunization on an increased delta OD, and the dark blue dog showed nine weeks post-immunization a peak of IgA against the homogenate from the (102-207)EgTrp biofilm. Further, did the two dogs receiving spores of the (370-583)EgA31 strain show a delta OD in the IgG and IgA ELISA, against the (102-207)EgTrp biofilm, one-week post-immunization until the fifth-week post-immunization. The Placebo dogs showed to have some IgA detectable in the ELISA against the (102-207)EgTrp biofilm. When analyzing

the serum samples in an ELISA against the (370-583)EgA31 biofilm, the delta OD for IgG revealed that the dog of the EgTrp group (bright blue bars) developed an IgG response in the last four weeks of the experiment.

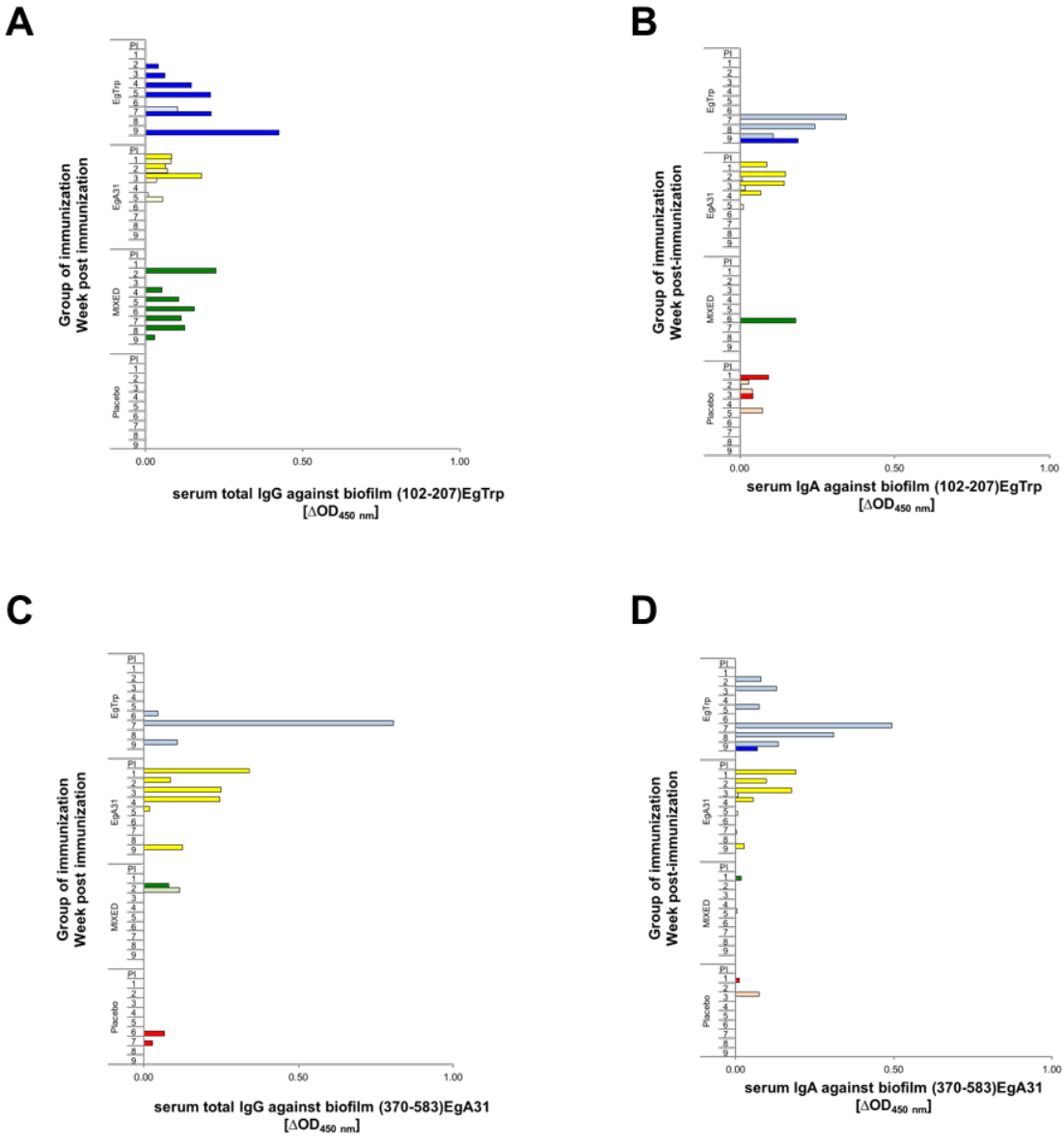


Figure 31: Humoral response of dogs immunized with recombinant *B. subtilis* spores, (102-207)EgTrp / (102-278)EgTrp, against recombinant *B. subtilis* biofilm.
Bar charts are showing the humoral immune response (total IgG and IgA) detected by indirect ELISA. The results were calculated by subtraction of the individual PI level of each dog. In blue (bright and dark) the EgTrp group, in yellow (bright and dark) the EgA31 group, in green (bright and dark) the Mixed group and in red (bright and dark) the Placebo group**. (A)** serum total IgG specific against homogenized (102-207)EgTrp biofilm. **(B)** serum IgA specific against homogenized (102-207)EgTrp biofilm. **(C)** serum total IgG specific against homogenized (370-583)EgA31 biofilm. **(D)** serum IgA specific against homogenized (370-583)EgA31 biofilm.

Further, did the dog of the EgA31 group (bright yellow bars) show an IgG response from one to five weeks post-immunization. When analyzing the IgA level detected in one dog of EgTrp group (light blue bars) showed from two till nine weeks post-immunization an elevated IgA level, peaking at seven weeks post-immunization. Whereas the IgA level of one of the dogs of the EgA31 group (light yellow bars) had a decreasing level of IgA from one-week post-immunization till four weeks post-immunization, reaching an undetectable level of IgA at five weeks post-immunization. When the results of the ELISA, against homogenates of the biofilms of the *B. subtilis* (102-207)EgTrp and the (370-583)EgA31 strains, were combined in an analysis that a signal in one ELISA but not in the other would represent a probably specific detection of the fusion protein. The results revealed that one dog of the EgTrp group (dark blue bars) developed an increasing IgG signal against the (102-207)EgTrp fusion protein, as no signal was detected when analyzing the serum samples of this dog in an ELISA against (370-583)EgA31. Further, did one dog of the mixed group (dark green bars) show an IgG response against the fusion protein TasA-EgTrp in the homogenized (102-207)EgTrp biofilm, as the serum sample did not contain a detectable amount of IgG against the homogenized biofilm (370-583)EgA31. However, one dog of the EgA31 group (bright yellow bars) showed an IgG and IgA reaction within both ELISA. Therefore, this detected IgG and IgA reaction could be against other antigenic proteins of the biofilm than only the TasA-fusion protein, or against the TasA component of the fusion protein and was counted therefore as non-specific against the TasA-(370-583)EgA31 antigen.

In summary, the result obtained in the ELISA testing blood serum samples of the dogs indicate that one of the immunized dogs (dark blue bars) with single spore for EgTrp antigen resulted positive for the tested recombinant antigens (H_6-TasA, H_6-EgTrp, and

H_6-EgA31) but not against H_6-mCherry. Further was an increasing signal against homogenized (102-207)EgTrp biofilm detected and no signal against the homogenized (370-583)EgA31 biofilm. Therefore, it remains unlighted against what component of the recombinant proteins the immune reaction was developed, but it is indicated that immunization against *B. subtilis* TasA-EgTrp could be detected in this dog. However, the other immunized dog (light blue bars) for EgTrp spores was not responding specifically due that similar immune response for this dog was observed for the control antigen H_6-mCherry. Also, the IgA immune response in those dogs was very low at late time post-immunization, which was consistent with the maturation of the constant region of the heavy chain from the immunoglobulin. On the other hand, when analyzing the immune response of one dog immunized with single spores for EgA31 (light yellow bars), the dog was responding either specifically against the TasA protein or other components of the *B. subtilis* biofilm as the dog showed a response against both tested homogenates of biofilms. In the case of the dogs immunized with the mixed spores for both antigens (EgTrp and EgA31), one of them (dark green bars) was responding specifically to the antigens from the biofilm extract from *B. subtilis* (102-207)EgTrp but not against the (370-583)EgA31 biofilm.

Blood-derived PBMC cellular immune response of the tested dogs

Further, to study the immunological state induced in these dogs, their cellular immune response was examined in the course of the immunization by stimulating T cells from peripheral blood monocyte cells (PBMC) derived from the weekly blood samples. In particular, $CD4^+$ cells and $CD8^+$ cells and T_{reg} cells ($CD4^+$, $CD25^{hi}$, $CTLA4^+$) populations were analyzed by flow cytometer after stimulation with recombinant H_6-EgTrp and H_6-EgA31 purified from *E. coli*.

This analysis was performed with the dogs of the EgA31, the mixed group, and one placebo dog.

Blood-derived T cells were stimulated using purified recombinant H_6-EgTrp and H_6-EgA31. The delta percentage of T-cells proliferating after being stimulated with the indicated antigen compared to the proliferation of the T cells stimulated by PMA as the positive control at the week post-immunization minus the values obtained pre-immune are presented in **figure 32**. The results revealed that the dog of the mixed group represented by the dark green bars developed an increased proliferation of $CD4^+$ cells when stimulated by H_6-EgTrp and H_6-EgA31. Whereas the $CD8^+$ cells were showing an increased proliferation when boosted by H_6-EgTrp after six weeks post immunization but

when excited by H_6-EgA31 an increased proliferation was detected from one-week post-immunization till eight weeks post-immunization. However, the values were inconsistent from week to week and showed to have a high variability. The second dog of the mixed group (bright green bars) showed an increased proliferation of $CD4^+$ cells when stimulated by H_6-EgTrp but not when the cells were boosted by H_6-EgA31. However, the $CD8^+$ cells were showing an increased proliferation when boosted by H_6-EgTrp and H_6-EgA31 but as well for this dog a high variability from week to week was detected. The dog receiving spores of the (370-583)EgA31 strain showed (dark yellow) to have $CD4^+$ and $CD8^+$ cells increasing the proliferation rate when stimulated by H_6-EgA31 but not when boosted by H_6-EgTrp.

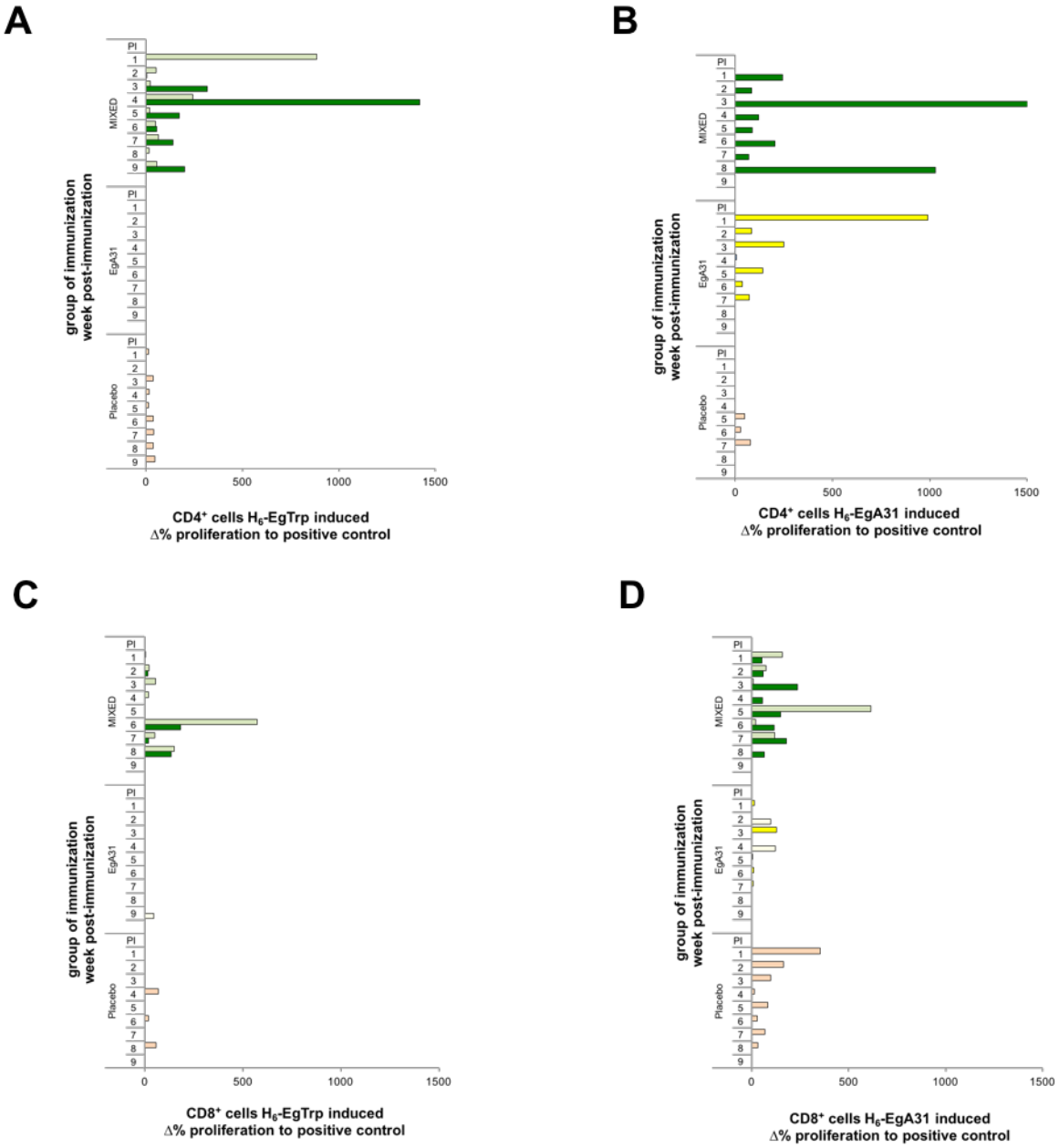


Figure 32: Cellular immune response tested for blood-derived PBMC of immunized dogs. Bar charts are showing the cellular immune response of blood derived PBMC detected by CFSE dilution and flow cytometer analysis. The results were calculated by subtraction of the individual PI level of each dog. In green (bright and dark) the Mixed group, in yellow (bright and dark) the EgA31 group and bright red the tested Placebo dog.
(A) $CD4^+$ PBMC stimulated by H_6-EgTrp. **(B)** $CD4^+$ PBMC stimulated by H_6-EgA31. **(C)** $CD8^+$ PBMC stimulated by H_6-EgTrp. **(D)** $CD8^+$ PBMC stimulated by H_6-EgA31

Further, it is to mention that the dog receiving the placebo did also show to have $CD4^+$ and $CD8^+$ cells increasing the proliferation rate when stimulated by H_6-EgTrp or H_6-EgA31. But for the $CD4^+$ cells stimulated by H_6-EgTrp or H_6-EgA31 and the $CD4^+$ cells boosted by H_6-EgTrp the proliferation was at a low level when comparing it to the cells derived from the other dogs. However, as the placebo dog was showing such a high response of $CD8^+$ cells when having stimulated the cells by H_6-EgA31, the response of $CD8^+$ cells of the other dogs shown in **Figure 32 D** was considered as background. Based on the inconsistent values measured for each dog and the partially high reactivity of the cells derived from the placebo dog it remains not-elucidated if the analysis is showing the particular reaction of the tested cells or the inconsistent reaction were an effect of an uncontrolled parameter of the experiment.

Additional were the blood derived T_{reg} PBMC cells ($CD4^+$, $CD25^{hi}$, $CTLA^+$) analyzed. Only in one of the EgA31 spore receiving dogs an increased proliferation of the T_{reg} cells was detected when having stimulated them by H_6-EgA31 (**Figure 33 B**). The increase in T_{reg} cell proliferation was detected four weeks post-immunization, which was coincident

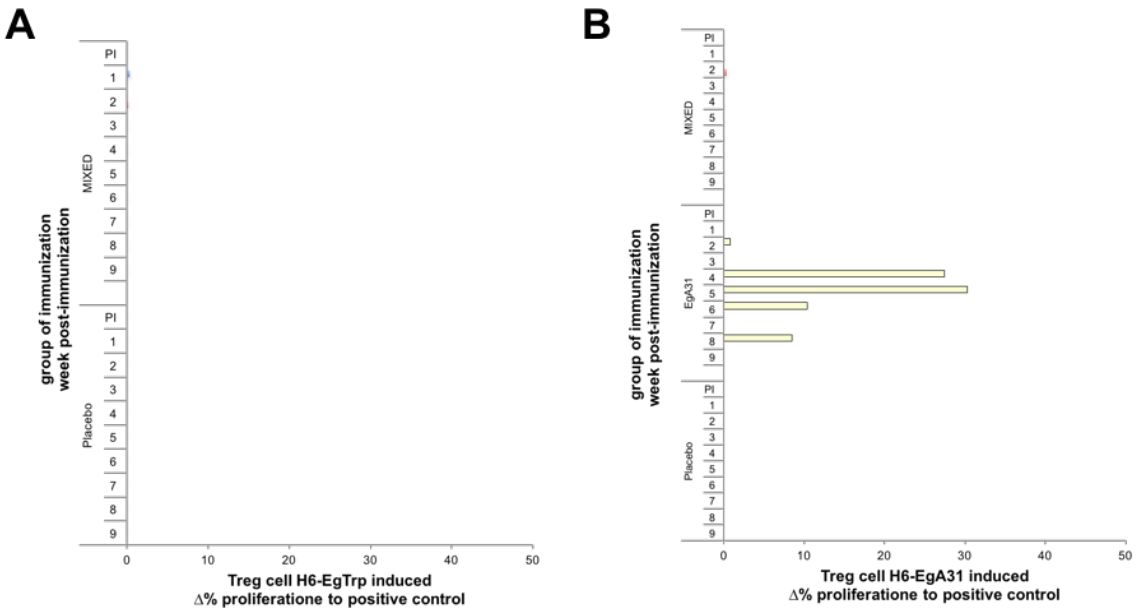


Figure 33: T regulatory cell proliferation induced by recombinant H_6-EgA31 of one of the EgA31 receiving dogs.
Bar charts are showing the cellular immune response of blood derived PBMC ($CD4^+$, $CD25^{hi}$, $CTLA^+$ T_{reg} cells) detected by CFSE dilution and flow cytometer analysis. The results were calculated by subtraction of the individual PI level of each dog. In yellow (bright and dark) the EgA31 group, in green (bright and dark) the Mixed group and bright red the tested Placebo dog**.**
(A) T_{reg} cells PBMC stimulated by H_6-EgTrp. **(B)** T_{reg} cells PBMC stimulated by H_6-EgA31

with the detected decrease of the $CD4^+$ T cells proliferation when being stimulated by H_6-EgA31.

As a manner to detect local cellular immunity, the derived T cells from the spleen, MLN and Peyer's patches were stimulated with recombinant purified H_6-EgTrp and H_6-EgA31. The results obtained were presented as a percentage of proliferation of the cells stimulated by the positive control (PMA induced). Therefore, rates of proliferation below 100 % were considered as non-responding. Remarkably $CD4^+$ cells derived from mesenteric lymph nodes (MLN) of dogs receiving spores of the EgTrp strain (bright blue and dark blue) were vigorously proliferating when stimulated by recombinant H_6-EgTrp, but only cells from one dog (bright blue) when the cells were derived from Peyer's patches. Different to that were $CD4^+$ cells derived from the spleen of the dogs receiving (370-583)EgA31 strain spores (yellow bright and dark) showing an increased proliferation when stimulated by H_6-EgA31. For the $CD8^+$ cells derived from MLN independent of the recombinant protein used and independent of the dog (placebo, EgTrp, and EgA31) showed an increased proliferation. However, $CD8^+$ spleen-derived cells of one dog of the mixed group (bright green) showed an increased proliferation for both stimuli (H_6-EgTrp and H_6-EgA31). However, the dog (dark green square) of the same group showed to have $CD8^+$ cells proliferating when derived from PP (H_6-EgTrp and H_6-EgA31 stimulated) and when stimulating by H_6-EgA31 also spleen-derived $CD8^+$ cells showed an increased proliferation for this dog. Interestingly one dog receiving EgTrp spores (dark blue) had an increase CD8+ proliferation derived from the spleen, whereas the other dog from the same group (bright blue) showed an increased CD8+ proliferation from $^+$Peyer Patches derived cells stimulated by H_6-EgTrp. Similarly to the dogs receiving (370-583)EgA31 spores (bright yellow) that have an increased $CD8^+$ proliferation of spleen-derived cells. Thus, the other dog of the same group (dark yellow) showed an increased CD8+proliferation Peyer Patches derived cells when H_6-EgA31 stimulated.

Based on the observation that the two dogs of the same group often appear to have different cellular immune response profiles (**Figure 34**) the interpretation of the results remains difficult and is further discussed in the discussion of this thesis.

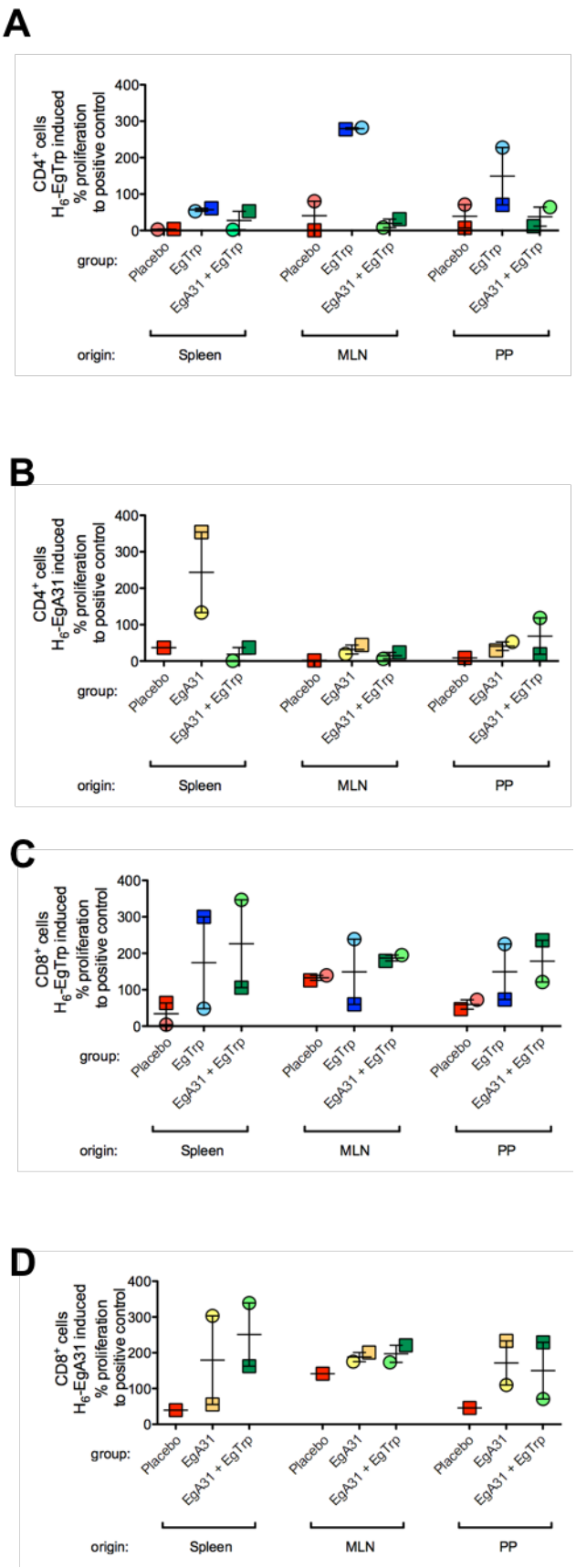


Figure 34: Cellular response of cells derived from the spleen, mesenteric lymph nodes and Peyer's patches of dogs immunized with recombinant *B. subtilis* spores carrying (102-207)EgTrp or (370-583)EgA31 antigen.

(A) Plot showing $CD4^+$proliferation induced by purified recombinant H_6-EgTrp. Each dot represents the mean percentage of maximal proliferation of one dog in triplicate. **(B)** Plot showing $CD4^+$ cellular proliferation induced by purified recombinant H_6-EgA31. **(C)** Plot showing $CD8^+$ cellular proliferation induced by purified recombinant H_6-EgTrp. **(D)** Plot showing $CD8^+$ cellular proliferation induced by purified recombinant H_6-EgA31. Each dot represents the mean percentage of maximal proliferation of one dog in triplicate.

For the plots (A) to (D) each dot corresponds to one dog by bright yellow (circle) and dark yellow (square) for EgA31, bright green (circle) and dark green (square) for the Mix bright red (circle) and dark red (square) for the Placebo. DPI, days post immunization; PI, pre-immune.

B. subtilis mutant bacteria extracted *ex vivo* of the intestine of immunized dogs

The small intestine from the dogs receiving the mixed spores for EgA31 and EgTrp was divided in duodenum, jejunum, and ileum. The intestinal content of each part was collected by perfusion and then cultivated in selective semi-solid media for the detection of recombinant *B. subtilis*. As denoted in **Table 5**, it was possible to isolate from the duodenum and the jejunum from one the dogs (corresponding to light green), 3200 and 12200 CFU/ml of the recombinant *B. subtilis*, respectively.

Dog	**Intestinal section**	**Estimated amount of bacteria**
Mix (light green)	Duodenum	3200 CFU/mL
	Jejunum	12200 CFU/mL
	Ileum	
Mix (dark green)	Duodenum	
	Jejunum	
	Ileum	

Table 5: CFU of recombinant *B. subtilis* detected in the small intestine of dogs receiving the mixture of EgTrp and EgA31 spores.

These colonies were analyzed by PCR using specific primers for detection of the *B. subtilis* EgTrp and EgA31 strains (**Figure 35 A and B**). The results showed the presence of both recombinant *B. subtilis* strains in duodenum and jejunum. Thereby, suggesting that spores from *B. subtilis* germinated and got establish in the small intestine of the inoculated dogs, in particular in the duodenum and jejunum. However, this analysis was performed with the mix group dogs but not with the other dogs. Further, as only one of both dogs showed to have a detectable amount of recombinant bacteria in the duodenum and the jejunum, this observation is left as a single result and not extrapolated any further.

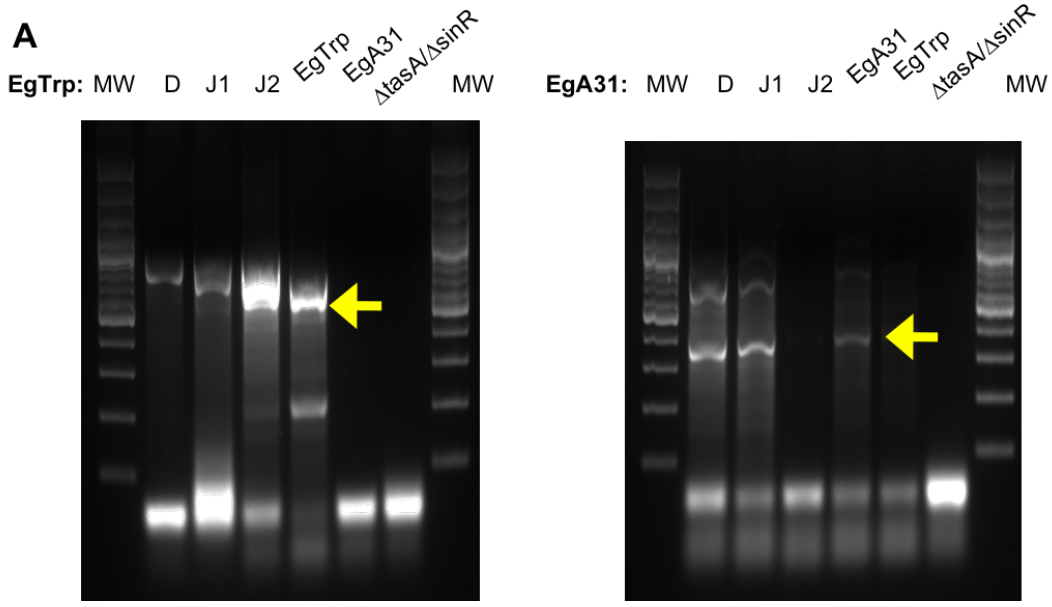


Figure 35: Intestinal content of one dog receiving the mixture of (102-207)EgTrp and (370-583)EgA31 showed to be for one dog recombinant EgA31 and EgTrp positive
End-point PCR analysis of the found bacteria in the intestine of the dog. The left panel is showing (102-207)EgTrp specific PCR and right panel showing (370-583)EgA31 specific PCR. D, J1, J2 describe the position of the found sample (D = duodenum, J = jejunum).

In conclusion of the dog experiment, I state that the body weight of the dogs was not influenced by the oral administration of *B. subtilis* recombinant spores. I observed indicators for a humoral immune response but also indicators for non-specific responses. Therefore, the humoral and cellular immune response do not give a clear answer if the recombinant spores would in a bigger population of dogs induce an immune response or not. Further, it is important that in at least one dog of the mixed group recombinant *B. subtilis* in the jejunum and ileum lumen were detected 18 days after the last administration of spores. Therefore, for this experimental setup performed with the analytical methods shown, revealed that one dog receiving recombinant *B. subtilis* (102-207)EgTrp (dark blue) most probably developed a humoral immune response specific against the *B. subtilis* (102-207)EgTrp biofilm.

Discussion

Why do we need an affordable vaccine against *E. granulosus?*

A successful vaccine needs to be effective in inducing a specific neutralizing immune response against the pathogen of interest, and furthermore, meet conditions as safety, production feasibility, affordable storage and stability over a reasonable time [22,204]. The *E. granulosus* is a significant burden in developing countries due to the absence of a licensed vaccine protecting the intermediate or the definitive host. A mathematical model developed by Torgerson et al. [173] suggested that targeting tapeworms in dogs could substantially decrease the parasite population. This model partially corroborated by treating dogs monthly with Praziquantel® (an antihelmintic drug), which reduced the rates of *E. granulosus* infection significantly in sheep being in close contact with the treated dogs [205]. However, due to the high frequency and the long duration of the eradication process for the *E. granulosus* tapeworms in a defined geographic area, the drug treatment resulted to be highly expensive and hard to manage. Therefore the use of a vaccine that could provide a long lasting protection in dogs would be highly advantageous [146,206,207]. However, a vaccine administrated subcutaneously or intramuscularly demands trained staff that restrains the animals [208], and also the vaccine has to be sterile and pH and osmolarity controlled, and be free from residual host proteins and DNA [209].

Previous studies demonstrated the ability of dogs to develop a specific immune response against *E. granulosus*, particularly against the protoscoleces membrane [177,210] and the secretory antigens [211,212]. Additionally, it has also been shown that the use of paramyosin [13,213–215] and tropomyosin [216,217] in immunization trials induced protective immunity against helminths infection. Thus, the paramyosin (EgA31) and tropomyosin (EgTrp) are detectable in the metacestodes and the protoscoleces states of *E. granulosus* [174]. When administrated to dogs in an immunization trial using a carrier as *Salmonella typhimurium* expressing EgTrp and EgA31 antigens, a 70% reduction in tapeworm number after challenging with the parasite is observed [13]. These promising results suggested that EgA31 and EgTrp could represent the antigens required for the eradication of *E. granulosus* in endemic areas. However, the use of attenuated *S. typhimurium* as carrier for enteric immunization has some drawbacks as are i) the expression of heterologous antigens is based on an episomic system, which can be lost by the bacteria; ii) the limited acceptance by the community of a pathogenic vector [218] and iii) reasonable lack of precedents regarding loss of the generated immunity after a natural infection with *S.*

typhimurium [219]. *B. subtilis,* in contrast to *S. typhimurium,* is in possession of a GRAS (generally regarded as safe) status. Also, recombinant *B. subtilis* spores had been already tested in several studies as a carrier for enteric antigens showing no adverse effect [23,220–222]. Thus, it suggests that *B. subtilis* is a good candidate for an enteric vaccine against *E. granulosus*. Also, numerous advantages are provided using spores of *B. subtilis* as an oral vaccine for human or veterinarian purposes. Some of these benefits correspond to general conditions of an oral vaccine as are: i) no special training of a specialized staff for the vaccine administration [223]; ii) in a veterinarian context, the animals do not require to be restrained as the vaccine can be administered directly within the food [208]. Additional specific features for this kind of vaccine are: i) the resistance to the stomach acidic environment [93]; ii) the lower impact for less-purified antigens [24,209]; iii) considered as safe [224]; iv) readily prepared and purified [27]; v) no requirement of a cold chain [93] and vi) the genetics and the physiology of *B. subtilis* is well-known. These conditions permit the development of a safe vaccine with controllable gene expression systems [26]. A major advantage of using spores of *B. subtilis,* as a carrier for an oral vaccine is the preparation and purification based in successive washes with distilled water (**see Materials and Methods**) that reduce the risk of chemical contamination. Also, the vaccines have a tendency to lose activity within a short time because of inactivation of the biological compounds, a process that is mainly temperature dependent [225]. In fact, currently only 31 of WHO licensed vaccines are stable enough to resist a longer exposure to room temperature [226]. Thus, the spores of *B. subtilis* are stable at room temperature and even can be frozen or exposed to temperatures as high as 45°C for 17 months without significant loss of effectiveness when tested in immunization trials [227,228]. The data presented in this thesis, clearly show that the recombinant *B. subtilis* spores that carry *E. granulosus* antigens EgTrp and EgA31 are succeeding all the resistance tests for spores (shelf-life, high temperatures, and low pH) (**Figure 12**). Thus, the working hypothesis that recombinant *B. subtilis* spores, compared to wild-type spores, would not be altered in their tenacity and shelf life at room temperature, was proven to be correct.

However, even showing similar resistance as the *B. subtilis* NCBI3610 spores, the number of spores of the recombinant strains was significantly reduced (**Figure 7E**). Previously, Serrano et al. [229] were pointing towards a role of TasA in the spore assembly as they observed a lack of the proper coat assembly in *B. subtilis* spores in a *tasA* deleted strain. In contrast, we find any impairment of the resistance of recombinant spores when demonstrating *tasA* (ΔtasA) or transducing it with TasA-*E. granulosus* antigens. Also, this observation is supported by transmitted electron microscopy images of the

recombinant *B. subtilis spores* showing no alterations of the spore coat structure. Thus, no change of the spore coat is observed neither in the absence of TasA (ΔtasA) nor with TasA fused to any of *E. granulosus* antigens ((102-207)EgTrp, (102-278)EgTrp or (370-583)EgA31). Collectively, the data suggest that the unaltered tenacity of recombinant spores does not depend on the lack of TasA. Also, the modification of the TasA structure fused to an antigenic peptide at its C-terminus seems not to have a role in spores resistance. However, the difference in the number of spores harvested from the wild type strain compared to the ΔtasA/ΔsinR strain is based on the impairment of the regulation of the spore formation, which is among other factors influenced by sinR [230].

The observations, support the hypothesis that the TasA-antigen is expressed *in vitro* in the structure of biofilms. In this line of thought, the results presented by Vlamakis et al. [183] showed that TasA is a major component of the ECM and influence on the biofilm phenotype. In this thesis, the presented immunization system contemplates that the antigen gets expressed on the surface of the biofilm, which in the gut intend to stimulate the GALT (Gut associated lymphoid tissue). By an *in vitro* system, we showed that TasA fused to *E. granulosus* antigen peptides gets expressed within the biofilm as denoted by antigen expression in immunoblotting using both anti-TasA and specific related peptidic antigen antibodies (**Figure 9 C and 10 C**). Also, immunohistochemistry of recombinant biofilms showed the localization of the antigen throughout the entire biofilm (**Figure 11**). The same results were obtained for all tested biofilm of the recombinant *B. subtilis* expressing *E. granulosus* antigenic peptides, EgTrp(102-207), EgTrp(102-278) and EgA31(370-583) strains. Moreover, the biofilm phenotype formed by the recombinant *B. subtilis* strains showed that the TasA-antigen fusion protein expression could compensate the phenotype architecture of the *B. subtilis* ΔtasA/ΔsinR strain (**Figure 9 B and 10 B**).

Are recombinant *B. subtilis* inducing an enteric immunity?

The basic strategy of a vaccine is the presentation of a determined antigen to the immune system, which gets then recognized as non-self and processed to induce an immune response when challenging by encountering a particular pathogen [15]. In the intestine, the antigen presenting cell (APC) are intestinal microfold (M) and dendritic cells (DCs), which are responsible for the capture, the transport, the processing and the presentation of the antigens [131]. The naïve B cells recognize the presented epitope exposed on the APCs by a specific surface IgM that gets stimulated by the gut-homing integrin α_4/β_7 and CCR9. These two cytokines are specific for the expression of the gut-associated mucosal directing cell adhesion molecule-1 (MAdCAM-1) and the TECK/CCL5 on T and B cells, respectively [132,231]. Thus, B and T cells primed in the

intestine to acquire distinct migration patterns to be directed to secondary lymphatic organs and the site of intestinal priming [131]. This immunological maturation leads to the generation of specific mature antibodies that recognize the first antigen, which can be detected in sera after 14 days and peak at 4 to 6 weeks from the primary immunization [232]. Only very few vaccines induce after a single immunization a high and sustained antibody response due to the limited antigen availability. Therefore, the immunization schedules usually include two to three inoculations, repeated in an interval of 3-4 weeks [233,234]. Consequently, the vaccination program used in this thesis was designed with three immunizations at day 1, 21 and 42 for both mice and dogs. Under these conditions in mouse experiments, the onset of detectable sIgA in feces started at day 42 post-immunization and raised until day 50. In the trials performed by Duc et al. [100], the administration of *B. subtilis* spores, which expressed intracellular ß-galactosidase when germinating, provided a sIgA in feces that peaked at day 58. However, the results of Duc et al., were obtained after nine oral gavages of spores. The time point of the onset of the mounted humoral immune response for specific EgTrp sIgA in feces was consistent in all mouse experiments when testing *B. subtilis* strains expressing (102-278)EgTrp independently of the treatment of antibiotics. The generated humoral immune response is limited to the local sIgA intestinal secretion [235]. The systemic humoral immune response was observed in the mouse experiments when administrating *B. subtilis* expressing (102-207)EgTrp after the antibiotics treatment (**Figure 20**). The stated hypotheses that recombinant *B. subtilis* spores can bypass the stomach and further germinate in the intestine of mice and dogs by expressing TasA fused to an antigenic protein, forming an intestinal biofilm can be answered only partially based on the obtained results. The recombinant *B. subtilis* spores can pass through the stomach of mice and dogs as the spores extracted out of the feces of the animals were still able to germinate (e.g. **Figure 14 D** and **Table 5**). Furthermore, the recombinant spores germinate in the intestine of mice and dogs as the humoral immune response against EgTrp(102-207), and EgTrp(102-278). However, the data presented is still not sufficient to proof that the germinated spores are expressing all component to form a biofilm or are only expressing the fusion protein TasA-EgTrp.

However, the detected humoral immune response was only significantly higher when compared to its pre-immune sample, but it was not significantly different from the detected humoral immune response in both placebo or *B. subtilis* background strain (ΔtasA/ΔsinR) groups. In contrast, the local humoral response of the (102-278)EgTrp mice group treated with antibiotics before each oral gavage (**Figure 24**). The antibiotics

treatment enable the humoral immune response against (102-207)EgTrp, likely by the opening of niches as result of a decrease in the taxonomic diversity in the intestinal microflora [125]. Studies from Brandl et al. [117] showed that treatments with a combination of antibiotics as vancomycin, metronidazole, and neomycin in mice lowered the intestinal expression of the antimicrobial peptide RegIII-γ, which is part of the mucosal innate immune defense [117]. Thus, through the reduction of the barrier function, it is possible that the *B. subtilis* (102-207)EgTrp strain enable the induction of a humoral immune response in mice. Also, the vancomycin treatment decrease the short intestinal chain fatty acids (SCFA) concentration, which correlates with a reduction of stimulated G-coupled receptor 43 (GPR43) in regulatory T (reg T) cells. Resulting in a reduction of Reg T cells population in the intestinal mucosa but not in other organs [236,237]. In our experimental approach, mice also received vancomycin among other antibiotics, which could lead to a reduction of the protective immunity having consequently the allowance of the recombinant *B. subtilis* spore germination. Thereby, the effects of an appropriate environmental condition for the spore germination are the enhancement of the TasA-EgTrp antigenic peptides that can be detected as a humoral immune response against the specific antigens. Also in mice, the systemic humoral response raised, as detect by the positive reaction of serum IgA against the EgTrp antigenic peptides presented by the recombinant *B. subtilis* strains. Meanwhile, the mice not receiving antibiotic treatment only developed a local humoral response (**Figure 15 A**). The humoral immune response obtained in the mouse model was consistent with the results obtained when testing the immune response in dogs to the recombinant *B. subtilis* spores. Thus, in mice a specific response against TasA-(102-207)EgTrp expressed in biofilm extract and from recombinant H_6-EgTrp but not against homogenates of *E. granulosus* protoscoleces (**Figure 16 and 21**).

The analysis the dog's serum showed an immune response against biofilm homogenized of two dogs (dark blue and dark green) immunized with recombinant *B. subtilis* spores carrying EgTrp an*B. subtilis*d (102-207)EgTrp strain, respectively (**Figure 31 A**). In a previous study performed by Zhang et al. [205], in which dogs were immunized three times subcutaneously with purified recombinant *E. granulosus* antigens in intervals of three weeks, showed a dog's humoral immune response after four weeks and lasted until the end of the experiment at 16 weeks. However, the immunization performed by Zhang et al. [205] raised a specific IgG response, whereas no IgA or IgE raise was detected. Even without the development of an IgA or IgE response, the dogs were protected (97 - 100%) concerning the suppression of growth and egg development in *E. granulosus* [205]. In

contrast, the rise of the dog serum IgG levels in our experimental set-up was already observed at two weeks after the first oral administration with recombinant spores. The humoral immune response was increasing until the end of the experiment, and it developed in IgA response (**Figure 31**), indicating that the immunization through oral administration in dogs seemed to be faster in generating a detectable immune response than subcutaneous immunization. The data in this work demonstrated that the recombinant spores induce the development of a humoral immune response in mice and dogs.

Another point was the absence of detectable humoral immune response against EgA31 antigenic peptides. The dogs and the mice receiving (370-583)EgA31 recombinant spores did not develop any specific increase of antibodies against (370-583)EgA31 in serum or feces. The two mouse experiments, in which were tested EgA31, were performed treating mice two and even three times with antibiotics in the drinking water. Even this antibiotic cocktail improve the response to (102-207)EgTrp, does not improve the mice for the recombinant EgA31 spores. The same results were observed in both mice and dogs experiments in which the recombinant (370-583)EgA31 spores. The negative response of the dogs and the mice towards the stimulation with EgA31 was probably based in no germination in GIT of the EgA31 spores of the dogs and mice or the development of tolerance against the EgA31 antigenic peptide. The first point can be argued against because of the EgTrp strains, possessing the same genetic background as the EgA31 strain, and can induce a humoral immune response in mice and hence germinated in the GIT. Also, the *in vitro* tests (biofilm phenotyping, expression by immunoblots, biofilm antigen localization by immunohistochemistry) did not show any particular impairment of the (370-583)EgA31 strain. Lastly, the spores detected in the duodenum and jejunum of one of the dogs which received a mixture of EgTrp and EgA31 spores, turned positive by PCR analysis when tested with the specific EgA31 primers, suggesting that (370-583)EgA31 spores were settled in the intestine of the dogs. Collectively the data suggest that the induction of tolerance in mice and dogs by EgA31 antigenic peptides could be responsible for the lack of humoral immune response to this antigen. This observation is sustained by a decrease in $CD4^+$ T cell proliferation, and an increase of regulatory T cell proliferation in one EgA31 immunized dogs (**Figure 32 and 33**). The results are consistent with previous reports, showing [238] that the T cell population is not only instructed by classic self/non-self discrimination mechanisms during thymic development, but is also educated in the periphery to accommodate the variety of non-self antigens derived from the commensal microbiota at mucosal sites. Thereby, leading to

tolerance by induction of T regulatory cells [238,239,240] Further, as the experimental results presented in this thesis pointed towards the stimulation of the regulatory T cells by TasA-(370-583)EgA31 due that serum regulatory T cell isolated from the dogs receiving the spore mixture (EgA31 and EgTrp) proliferate after stimulation by H_6-EgA31 indicating the development specific tolerance toward EgA31 antigenic peptide. On the other hand, the dogs receiving recombinant spores showed a higher level of T cells derived from PBMC, spleen, mesenteric lymph nodes and Peyer's patches than placebo dogs. The dogs administered with the mixture of spores showed proliferation of $CD8^+$ T cells derived from Peyer's patches when stimulated with recombinant purified H_6-EgTrp and H_6-EgA31. Commonly, the stimulation of $CD8^+$ T cells is trigger by the presentation of intracellular antigens in an MHC class I context by the APCs, as are for example the viral proteins. However, exogenous antigens can also lead to a stimulation of $CD8^+$ T cells [241].
As $CD8^+$ T cells need stimulation through antigen-presenting cells (APC), the APC when not directly infected need to acquire exogenous antigens from and present them on MHC class I molecules, by cross-presentation. Dendritic cells show to have a more regulated phagocytic pathway that serves antigen processing and presentation on MHC molecules compared to macrophages and neutrophils that mainly destroy antigens. Dendritic cells are capable of processing exogenous antigens and to cross-present them on MHC I to $CD8^+$ cells [242].

In conclusion of this work, recombinant *B. subtilis* spores were engineered to express within the biofilms the antigenic peptides TasA-EgTrp and TasA-EgA31 protein. The tenacity of the recombinant spores was not altered when compared to *B. subtilis* wild-type spores as denoted by their shelf-life, temperature resistance and resistance to low pH. The spores were able to germinate after the passage through the stomach, as the recombinant *B. subtilis* spores were detected after the oral gavage in the feces of mice and intestinal content of dogs. Even if a direct evidence for the intestinal germination and biofilm formation is not determined during the present work, indirect evidence suggests that this is the point. Thus, the mounted specific immune response in dogs and mice against the TasA-EgTrp, strongly indicating the presence of recombinant vegetative *B. subtilis* cells in the intestinal tract.
The evidence presented in here also showed that the nature of the antigen exposed in the biofilm could possibly induce tolerance. As is the case for EgA31 antigenic peptide, which showed no proliferation of T cells in mice but was detected in the microflora of one dog but been unable to induce a humoral immune response in both dogs and mice.

Therefore, it remains the last working hypothesis of this work without proof. The oral administration of a combined dose of recombinant *B. subtilis* spores for EgTrp and EgA31 did not induce exacerbated humoral and cellular immune response detectable by the methods applied.

A proof of principle for the successful oral vaccination of dogs against *E. granulosus* by recombinant *B. subtilis* would depend on a direct challenge of dogs with viable *E. granulosus* protoscoleces. It is expected to detect a significant reduction in the *E. granulosus* worm burden among the group of recombinant spores immunized dogs compared with the group of unimmunized dogs. Unfortunately, due to biosafety concerns, this kind of experiment cannot be performed at the animal facility of the University of Zurich. The UZH facility is not in possession of a BL-3 biohazard safety level for dog experimentation, as stipulated by the Swiss legislation when working with definitive host infected in a mature state or eggs from *Echinococcus* spp [243,244]. However, a challenge is possible through our collaborators from Tunisia and Morocco. As described above, there are hints that recombinant spores carrying EgA31 antigenic peptide induce tolerance; it will be of general interest to corroborate the tolerogenic effect of this particular antigen through an *in vivo* challenge. Deplazes et al., 1995 [245] described in a dog experimental trial that ovalbumin when orally administered in high amounts induces tolerance. Thus, to determine the tolerogenic potential of the *B. subtilis* vaccination system, dogs or mice immunized against ovalbumin (OVA) can be orally treated with recombinant spores expressing TasA-OVA to determine the immune reaction of the treated animals. Indicators for a tolerance would be downregulation of inflammation factors as are for example interleukin 4 (IL-4) (macrophage activation) [246] and interferon-γ (IFN-γ) (enhancing phagocytosis of neutrophils and the formation of reactive oxygen species) [247] as well as an increase of regulatory T cells [248]. The induction of tolerance is essential for the treatment of several autoimmune diseases (as Hashimoto's thyroiditis, Grave's disease, Type 1 diabetes, Primary biliary cirrhosis) making valuable the deepest insight in this research area. Perhaps the recombinant *B. subtilis* spores can be a good alternative for further treatments [249].

On the other hand, recombinant spores carrying EgTrp antigenic peptides were able to stimulate, but it could not be elucidated how specific, the local immune response. This aspect of the stimulation of the local immune system of the GIT can be very advantageous. For example for when required of local anti-TNFα antibodies in the treatment of chronic granulomatous disease [250] or to generate protection against enteric

diseases as rotavirus, coronavirus or even norovirus. Thereby, a clear insight of each antigen used as immunogen has to be scrutinized to determine if it is carrying tolerogenic or stimulating properties

An interesting approach could be to prolong the retention time of the recombinant spores within the GIT. Therefore, recombinant spores expressing epithelial or mucosal adherence factors on their surface, as recently developed by Batista et al. [222] could be useful.

Finally, by developing a vaccine based in spores of recombinant *B. subtilis* has many advantages, in particular by the expression of the antigens as C-terminal fusions to the matrix protein TasA. This idea was developed for the first time during this thesis work, and it is a promising manner for antigen presentation to the host and induction of local immunity.

References

1. Hopkins, D. R. The greatest killer: smallpox in history. (1983).
2. Thomas, H. The Peloponnesian War; Thucydes. (1959).
3. Behbehani, A. M. The smallpox story: life and death of an old disease. Microbiol. Rev. **47,** 455–509 (1983).
4. Sánchez-Sampedro, L. et al. The Evolution of Poxvirus Vaccines. Viruses **7,** 1726–1803 (2015).
5. McVey, S. & Shi, J. Vaccines in Veterinary Medicine: A Brief Review of History and Technology. Vet. Clin. North Am. Small Anim. Pract. **40,** 381–392 (2010).
6. Lombard, M., Pastoret, P. P. & Moulin, a M. A brief history of vaccines and vaccination. Rev. Sci. Tech. **26,** 29–48 (2007).
7. Albert, S. B. Oral Poliovirus Vaccine: History of Its Development and Use and Current Challenge to Eliminate Poliomyelitis from the World. J. Infect. Dis. (1985).
8. Lawson, K. F., Walker, V. C. R. & Crawley, J. F. Modified Swine Erysipelas Vaccine. (1958).
9. Nabel, G. J. Designing Tomorrow's Vaccines. N. Engl. J. Med. **368,** 551–560 (2013).
10. Wei, C.-J. Induction of Broadly Neutralizing H1N1. Science (80-.). **329,** 1060–1064 (2010).
11. Ledgerwood, J. E. et al. DNA priming and influenza vaccine immunogenicity: two phase 1 open label randomised clinical trials. Lancet Infect. Dis. **11,** 916–924 (2011).
12. Niu, B. L. et al. Myeloid dendritic cells loaded with dendritic tandem multiple antigenic telomerase reverse transcriptase (hTERT) epitope peptides: A potentially promising tumor vaccine. Vaccine **30,** 3395–3404 (2012).
13. Petavy, A.-F. et al. An oral recombinant vaccine in dogs against *Echinococcus granulosus*, the causative agent of human hydatid disease: a pilot study. PLoS Negl. Trop. Dis. **2,** e125 (2008).
14. Vesikari, T. et al. Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study. Lancet **370,** 1757–1763 (2007).
15. Huang, J. M. et al. Mucosal delivery of antigens using adsorption to bacterial spores. Vaccine **28,** 1021–1030 (2010).
16. SwissMedic. Swissmedic List of Permitted Vaccines. **31.10.2015,** (2015).
17. IVI. Institute of Virology and Immunology List of Permitted Animal Vaccines. (2015). at <http://www.blv.admin.ch/ivi/01739/01740/01773/index.html?lang=de>
18. Saleem, A. F. et al. Immunogenicity of poliovirus vaccines in chronically malnourished infants: A randomized controlled trial in Pakistan. Vaccine **33,** 2757–2763 (2015).
19. Zhang, W. & McManus, D. P. Vaccination of dogs against *Echinococcus granulosus:* a means to control hydatid disease? Trends Parasitol. **24,** 419–424 (2008).
20. Duthie, M. S., Gillis, T. P. & Reed, S. G. Advances and hurdles on the way toward a leprosy vaccine. Hum. Vaccin. **7,** 1172–83 (2011).
21. Bethony, J. M. et al. Vaccines to combat the neglected tropical diseases. Immunol. Rev. **239,** 237–270 (2011).
22. Saroja, C., Lakshmi, P. & Bhaskaran, S. Recent trends in vaccine delivery systems: A review. Int. J. Pharm. Investig. **1,** 64–74 (2011).
23. FDA. GRAS Notice 000562: *Bacillus subtilis*. (2014).
24. Amuguni, H. & Tzipori, S. *Bacillus subtilis*: a temperature resistant and needle free delivery system of immunogens. Hum. Vaccin. Immunother. **8,** 979–986 (2012).
25. Setlow, P. Spores of *Bacillus subtilis*: Their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. **101,** 514–525 (2006).
26. Ferreira, L. C. S., Ferreira, R. C. C. & Schumann, W. *Bacillus subtilis* as a tool for vaccine development: From antigen factories to delivery vectors. An. Acad. Bras. Cienc.

77, 113–124 (2005).
27. Tavares, M. B. et al. *Bacillus subtilis* endospores at high purity and recovery yields: optimization of growth conditions and purification method. Curr. Microbiol. **66,** 279–85 (2013).
28. Ciabattini, A., Parigi, R., Isticato, R., Oggioni, M. R. & Pozzi, G. Oral priming of mice by recombinant spores of *Bacillus subtilis.* Vaccine **22,** 4139–4143 (2004).
29. Nicholson, W. L. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. **59,** 410–416 (2002).
30. Hong, H. A., Duc, L. H. & Cutting, S. M. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. **29,** 813–35 (2005).
31. Tomohiro, H., Akio, A., Kan, K. & Shuichi, K. Changes in fecal microflora induced by intubation of mice with *Bacillus subtilis (natto)* spores are dependent upon dietary components. Can. J. Microbiol.
32. O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm formation as microbial development. Annu. Rev. Microbiol. **54,** 49–79 (2000).
33. Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. **56,** 187–209 (2002).
34. Chai, L. et al. Isolation, characterization, and aggregation of a structured bacterial matrix precursor. J. Biol. Chem. **288,** 17559–17568 (2013).
35. Branda, S. S., Chu, F., Kearns, D. B., Losick, R. & Kolter, R. A major protein component of the *Bacillus subtilis* biofilm matrix. Mol. Microbiol. **59,** 1229–38 (2006).
36. Diggle, S. P. et al. The galactophilic lectin, LecA, contributes to biofilm development in Pseudomonas aeruginosa. Environ. Microbiol. **8,** 1095–1104 (2006).
37. Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W. & Römling, U. The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix. Mol. Microbiol. **39,** 1452–1463 (2001).
38. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. Extracellular DNA required for bacterial biofilm formation. Science **295,** 1487 (2002).
39. Conrad, A. et al. Fatty acids of lipid fractions in extracellular polymeric substances of activated sludge flocs. Lipids **38,** 1093–1105 (2003).
40. Flemming, H.-C. & Wingender, J. The biofilm matrix. Nat. Rev. Microbiol. **8,** 623–33 (2010).
41. Iii, M. C. W. et al. Contributions of Antibiotic Penetration , Oxygen Limitation , and Low Metabolic Activity to Tolerance of *Pseudomonas aeruginosa* Biofilms to Ciprofloxacin and Tobramycin Contributions of Antibiotic Penetration , Oxygen Limitation , and Low Metabolic Activi. **47,** 317–323 (2003).
42. Jefferson, K. K., Goldmann, D. a. & Pier, G. B. Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. Antimicrob. Agents Chemother. **49,** 2467–2473 (2005).
43. Mai-Prochnow, A. et al. Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several gram-negative bacteria. J. Bacteriol. **190,** 5493–5501 (2008).
44. Vuong, C. et al. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell. Microbiol. **6,** 269–275 (2004).
45. Koch, C. & Hoiby, N. Pathogenesis of cystic fibrosis. Lancet **341,** 1065–1069 (1993).
46. Govan, J. R. & Deretic, V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol. Rev. **60,** 539–574 (1996).
47. Foxman, B. The epidemiology of urinary tract infection. Nat. Rev. Urol. **7,** 653–660 (2010).
48. Tsoligkas, A. N. et al. Engineering Biofilms for Biocatalysis. ChemBioChem **12,** 1391–1395 (2011).
49. Sundar, K., Sadiq, I. M., Mukherjee, A. & Chandrasekaran, N. Bioremoval of trivalent

chromium using *Bacillus* biofilms through continuous flow reactor. J. Hazard. Mater. **196,** 44–51 (2011).
50. Branda, S. S., González-Pastor, J. E., Ben-Yehuda, S., Losick, R. & Kolter, R. Fruiting body formation by *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S. A. **98,** 11621–11626 (2001).
51. Stöver, A. G. & Driks, A. Secretion, localization, and antibacterial activity of TasA, a Bacillus subtilis spore-associated protein. J. Bacteriol. **181,** 1664–1672 (1999).
52. Romero, D., Aguilar, C., Losick, R. & Kolter, R. Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. Proc. Natl. Acad. Sci. U. S. A. **107,** 2230–2234 (2010).
53. Romero, D., Vlamakis, H., Losick, R. & Kolter, R. An accessory protein required for anchoring and assembly of amyloid fibres in *B. subtilis* biofilms. Mol. Microbiol. **80,** 1155–1168 (2011).
54. Stragier, P. & Losick, R. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30,** 297–241 (1996).
55. Fujita, M. & Losick, R. Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. Genes Dev. **19,** 2236–44 (2005).
56. Jiang, M., Shao, W., Perego, M. & Hoch, J. A. Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. Mol. Microbiol. **38,** 535–42 (2000).
57. Kearns, D. B., Chu, F., Branda, S. S., Kolter, R. & Losick, R. A master regulator for biofilm formation by *Bacillus subtilis*. Mol. Microbiol. **55,** 739–49 (2005).
58. Kobayashi, K. Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis.* Mol. Microbiol. **66,** 395–409 (2007).
59. Chai, Y., Kolter, R. & Losick, R. Paralogous Antidepressors Acting on the Master Regulator for Biofilm Formation in *Bacillus subtilis*. **42,** 157–162 (2008).
60. Mielich-Süss, B. & Lopez, D. Molecular mechanisms involved in *Bacillus subtilis* biofilm formation. Environ. Microbiol. **17,** 555–565 (2015).
61. Cairns, L. S., Hobley, L. & Stanley-Wall, N. R. Biofilm formation by *Bacillus subtilis* : new insights into regulatory strategies and assembly mechanisms. Mol. Microbiol. **93,** 587–598 (2014).
62. Lemon, K. P., Earl, A. M., Vlamakis, H. C., Aguilar, C. & Kolter, R. Biofilm development with an emphasis on *Bacillus subtilis*. Curr. Top. Microbiol. Immunol. **322,** 1–16 (2008).
63. Kennedy, M. J., Reader, S. L. & Swierczynski, L. M. Preservation records of micro-organisms: Evidence of the tenacity of life. Microbiology **140,** 2513–2529 (1994).
64. Cano, R. J. & Borucki, M. K. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science **268,** 1060–1064 (1995).
65. Errington, J. Regulation of endospore formation in *Bacillus subtilis*. Nat. Rev. Microbiol. **1,** 117–126 (2003).
66. Piggot, P. J. & Hilbert, D. W. Sporulation of *Bacillus subtilis*. Curr. Opin. Microbiol. **7,** 579–586 (2004).
67. Tan, I. S. & Ramamurthi, K. S. Spore formation in *Bacillus subtilis*. Environ. Microbiol. Rep. **6,** 212–225 (2014).
68. Paredes-Sabja, D., Setlow, P. & Sarker, M. R. Germination of spores of *Bacillales* and *Clostridiales* species: Mechanisms and proteins involved. Trends Microbiol. **19,** 85–94 (2011).
69. Piggot, P. J. & Coote, J. G. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40,** 908–962 (1976).
70. Veening, J.-W. et al. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc. Natl. Acad. Sci. U. S. A. **105,** 4393–4398 (2008).

71. Molle, V. et al. The Spo0A regulon of *Bacillus subtilis*. Mol. Microbiol. **50,** 1683–1701 (2003).
72. Chai, Y., Kolter, R. & Losick, R. Reversal of an epigenetic switch governing cell chaining in *Bacillus subtilis* by protein instability. Mol. Microbiol. **78,** 218–229 (2010).
73. Boonstra, M. et al. Spo0A regulates chromosome copy number during sporulation by directly binding to the origin of replication in *Bacillus subtilis*. Mol. Microbiol. **87,** 925–38 (2013).
74. Perry, S. E. & Edwards, D. H. The *Bacillus subtilis* DivIVA Protein Has a Sporulation-Specific Proximity to Spo0J. J. Bacteriol. **188,** 6039–6043 (2006).
75. Rahn-Lee, L., Merrikh, H., Grossman, A. D. & Losick, R. The sporulation protein SirA inhibits the binding of DnaA to the origin of replication by contacting a patch of clustered amino acids. J. Bacteriol. **193,** 1302–7 (2011).
76. Veening, J.-W., Murray, H. & Errington, J. A mechanism for cell cycle regulation of sporulation initiation in *Bacillus subtilis*. Genes Dev. **23,** 1959–70 (2009).
77. Wu, L. J. & Errington, J. *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. Science **264,** 572–575 (1994).
78. Henriques, A. O. & Moran, C. P. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. **61,** 555–588 (2007).
79. McKenney, P. T., Driks, A. & Eichenberger, P. The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. Nat. Rev. Microbiol. **11,** 33–44 (2013).
80. Leggett, M. J., Mcdonnell, G., Denyer, S. P., Setlow, P. & Maillard, J. Y. Bacterial spore structures and their protective role in biocide resistance. J. Appl. Microbiol. **113,** 485–498 (2012).
81. Gould, G. W. History of science - Spores: Lewis B Perry Memorial Lecture 2005. J. Appl. Microbiol. **101,** 507–513 (2006).
82. Setlow, P. Spore germination. Curr. Opin. Microbiol. **6,** 550–556 (2003).
83. Driks, A. *Bacillus subtilis* Spore Coat. Microbiol. Mol. Biol. Rev. **63,** 1–20 (1999).
84. Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. **64,** 548–72 (2000).
85. Paidhungat, M., Setlow, B. & Driks, A. Characterization of Spores of *Bacillus subtilis* Which Lack Dipicolinic Acid. **182,** 5505–5512 (2000).
86. Lai, E. et al. Proteomic Analysis of the Spore Coats of *Bacillus subtilis* and *Bacillus anthracis.* Society **185,** 1443–1454 (2003).
87. Popham, D. L. Specialized peptidoglycan of the bacterial endospore: The inner wall of the lockbox. Cell. Mol. Life Sci. **59,** 426–433 (2002).
88. Cortezzo, D. E. & Setlow, P. Analysis of factors that influence the sensitivity of spores of Bacillus subtilis to DNA damaging chemicals. J. Appl. Microbiol. **98,** 606–617 (2005).
89. Cowan, A. E., Koppel, D. E., Setlow, B. & Setlow, P. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. Proc. Natl. Acad. Sci. U. S. A. **100,** 4209–4214 (2003).
90. Douki, T., Setlow, B. & Setlow, P. Photosensitization of DNA by dipicolinic acid, a major component of spores of *Bacillus* species. Photochem. Photobiol. Sci. **4,** 591–597 (2005).
91. Casula, G. & Cutting, S. M. Bacillus Probiotics: Spore Germination in the Gastrointestinal Tract. Appl. Environ. Microbiol. **68,** 2344–2352 (2002).
92. Duc, L. H., Hong, H. a., Uyen, N. Q. & Cutting, S. M. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine **22,** 1873–1885 (2004).
93. Duc, L. H., Hong, H. a. & Cutting, S. M. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. Vaccine **21,** 4215–4224 (2003).
94. La Ragione, R. M., Casula, G., Cutting, S. M. & Woodward, M. J. *Bacillus subtilis* spores competitively exclude Escherichia coli O78:K80 in poultry. Vet. Microbiol. **79,** 133–42

(2001).
95. La Ragione, R. M. & Woodward, M. J. Competitive exclusion by *Bacillus subtilis* spores of Salmonella enterica serotype Enteritidis and Clostridium perfringens in young chickens. Vet. Microbiol. **94,** 245–56 (2003).
96. Le Duc, H., Hong, H. a, Barbosa, T. M., Henriques, A. O. & Cutting, S. M. Characterization of *Bacillus* Probiotics Available for Human Use. Appl. Environ. Microbiol. **70,** 2161–2171 (2004).
97. Ceragioli, M. et al. Phagocytosis, germination and killing of *Bacillus subtilis* spores presenting heterologous antigens in human macrophages. Microbiology **155,** 338–346 (2009).
98. Hoa, T. T. et al. Fate and Dissemination of *Bacillus subtilis* Spores in a Murine Model. Appl. Environ. Microbiol. **67,** 3819–3823 (2001).
99. Hong, H. A. et al. *Bacillus subtilis* isolated from the human gastrointestinal tract. Res. Microbiol. **160,** 134–143 (2009).
100. Duc, L. H., Hong, H. a., Fairweather, N., Ricca, E. & Cutting, S. M. Bacterial spores as vaccine vehicles. Infect. Immun. **71,** 2810–2818 (2003).
101. D'Arienzo, R. et al. *Bacillus subtilis* spores reduce susceptibility to Citrobacter rodentium-mediated enteropathy in a mouse model. Res. Microbiol. **157,** 891–7 (2006).
102. Fuller, R. Probiotics in man and animals. J. Appl. Bacteriol. **66,** 365–78 (1989).
103. Fuller, R. Probiotics in human medicine. Gut **32,** 439–442 (1991).
104. Schneitz, C. & Hakkinen, M. Comparison of two different types of competitive exclusion products. Lett Appl Microbiol **26,** 338–341 (1998).
105. Endo, T., Nakano, M., Shimizu, S., Fukushima, M. & Miyoshi, S. Effects of a probiotic on the lipid metabolism of cocks fed on a cholesterol-enriched diet. Biosci. Biotechnol. Biochem. **63,** 1569–1575 (1999).
106. Fukushima, M. & Nakano, M. Effects of a mixture of organims, *Lactobacillus acidophilus* or *Streptococcus faecalis* on cholesterol in rats fed on a fat- and cholesterol-enriched diet. Br. J. Nutr. **76,** 857–867 (1996).
107. Cenci, G., Rossi, J., Trotta, F. & Caldini, G. Lactic acid bacteria isolated from dairy products inhibit genotoxic effect of 4-nitroquinoline-1-oxide in SOS-chromotest. Syst. Appl. Microbiol. **25,** 483–490 (2002).
108. Rhee, K.-J., Sethupathi, P., Driks, A., Lanning, D. K. & Knight, K. L. Role of Commensal Bacteria in Development of Gut-Associated Lymphoid Tissues and Preimmune Antibody Repertoire. J. Immunol. **172,** 1118–1124 (2004).
109. Hong, P.-Y., Wheeler, E., Cann, I. K. O. & Mackie, R. I. Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galápagos Islands using 16S rRNA-based pyrosequencing. ISME J. **5,** 1461–1470 (2011).
110. Engel, P. & Moran, N. A. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol. Rev. **37,** 699–735 (2013).
111. Cerf-Bensussan, N. & Gaboriau-Routhiau, V. The immune system and the gut microbiota: friends or foes? Nat. Rev. Immunol. **10,** 735–744 (2010).
112. Hooper, L. V., Midtvedt, T. & Gordon, J. I. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. **22,** 283–307 (2002).
113. Vollaard, E. J. & Clasener, H. a L. Colonization resistance. Antimicrob. Agents Chemother. **38,** 409–414 (1994).
114. Koeth, R. A. et al. Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat. Med. **19,** 576–585 (2013).
115. Ramakrishna, B. S. Role of the gut microbiota in human nutrition and metabolism. J. Gastroenterol. Hepatol. **28,** 9–17 (2013).
116. Hill, D. a. & Artis, D. Intestinal Bacteria and the Regulation of Immune Cell Homeostasis. Annu. Rev. Immunol. **28,** 623–667 (2010).
117. Brandl, K. et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature **455,** 804–7 (2008).

118. Hill, D. a et al. Commensal bacteria–derived signals regulate basophil hematopoiesis and allergic inflammation. Nat. Med. **18,** 538–546 (2012).
119. Abraham, C. & Cho, J. H. Inflammatory Bowel Disease. N. Engl. J. Med. 2066–2078 (2009). doi:10.1056/NEJMra0804647
120. Wen, L. et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature **455,** 1109–1113 (2008).
121. Wu, H.-J. et al. Gut-Residing Segmented Filamentous Bacteria Drive Autoimmune Arthritis via T Helper 17 Cells. Immunity **32,** 815–827 (2010).
122. Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology **156,** 3216–3223 (2010).
123. Ubeda, C. & Eric G Pamer. Antibiotics, microbiota, and immune defense. **42,** 157–162 (2012).
124. Huse, S. M. et al. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Genet. **4,** (2008).
125. Dethlefsen, L. & Relman, D. A. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. **108,** 4554–4561 (2010).
126. Ubeda, C. et al. Vancomycin-resistantant *Enterococcus* domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. Conflict **120,** (2010).
127. Wlodarska, M. et al. Antibiotic Treatment Alters the Colonic Mucus Layer and Predisposes the Host to Exacerbated *Citrobacter rodentium*-Induced Colitis. Infect. Immun. **79,** 1536–1545 (2011).
128. Bouskra, D. et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature **456,** 507–510 (2008).
129. Clarke, T. B. et al. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. **16,** 228–231 (2010).
130. Hill, D. a et al. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. **3,** 148–158 (2010).
131. Marcela F. Pasetti, Jakub K. Simon, Marcelo B. Sztein, M. M. L. Immunology of Gut Mucosal Vaccines. Immunol. Rev. **29,** 997–1003 (2012).
132. Soloff, A. C. & Barratt-Boyes, S. M. Enemy at the gates: dendritic cells and immunity to mucosal pathogens. Cell Res. **20,** 872–885 (2010).
133. Cerutti, a. Location, location, location: B-cell differentiation in the gut lamina propria. Mucosal Immunol. **1,** 8–10 (2008).
134. Macpherson, A. J. et al. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science **288,** 2222–2226 (2000).
135. Brandtzaeg, P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine **25,** 5467–5484 (2007).
136. Macpherson, A. J., McCoy, K. D., Johansen, F.-E. & Brandtzaeg, P. The immune geography of IgA induction and function. Mucosal Immunol. **1,** 11–22 (2008).
137. Boullier, S. et al. Secretory IgA-mediated neutralization of *Shigella* flexneri prevents intestinal tissue destruction by down-regulating inflammatory circuits. J. Immunol. **183,** 5879–5885 (2009).
138. Cáceres, V. M. & Sutter, R. W. Sabin monovalent oral polio vaccines: review of past experiences and their potential use after polio eradication. Clin. Infect. Dis. **33,** 531–541 (2001).
139. Grassly, N. C. et al. Protective efficacy of a monovalent oral type 1 poliovirus vaccine: a case-control study. Lancet **369,** 1356–1362 (2007).
140. Jafari, H. et al. Polio eradication. Efficacy of inactivated poliovirus vaccine in India. Science **345,** 922–5 (2014).
141. El-Sayed, N. et al. Survey of poliovirus antibodies during the final stage of polio

eradication in Egypt. Vaccine **25,** 5062–5070 (2007).
142. Caidi, H., Bennis, F. & Mouan, N. contre la poliomyélite et la rougeole chez les enfants malnutris au Maroc. **10,** 474–481 (2004).
143. Center for Disease Control and Prevention. Notes from the field: Severe illness associated with synthetic cannabinoid use - Brunswick, Georgia, 2013. MMWR Morb Mortal Weekly Report **62,** (2013).
144. Who. Accelerating Work To Overcome the Global Impact of Neglected Tropical. (2012).
145. Zheng, H. et al. The genome of the hydatid tapeworm *Echinococcus granulosus*. Nat. Genet. **45,** 1168–75 (2013).
146. Grosso, G., Gruttadauria, S., Biondi, A., Marventano, S. & Mistretta, A. Worldwide epidemiology of liver hydatidosis including the Mediterranean area. World J. Gastroenterol. **18,** 1425–1437 (2012).
147. Zhang, W., Li, J. & McManus, D. P. Concepts in immunology and diagnosis of hydatid disease. Clin. Microbiol. Rev. **16,** 18–36 (2003).
148. Koziol, U. & Brehm, K. Recent advances in *Echinococcus* genomics and stem cell research. Vet. Parasitol. (2015). doi:10.1016/j.vetpar.2015.07.031
149. Craig, P. et al. Prevention and control of cystic echinococcosis. **7,** 385–394 (2007).
150. WHO/OIE. Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern. Veterinary Parasitology **104,** (2002).
151. R. C. a. Thompson, Kumaratilake, L. M. & Eckert, J. Observations on *Echinococcus Granulosus* of Cattle Origin in Switzerland. Int. J. Parasitol. **14,** 283–291 (1984).
152. Haag, K. L., Gottstein, B. & Ayala, F. J. Taeniid history, natural selection and antigenic diversity: evolutionary theory meets helminthology. Trends Parasitol. **24,** 96–102 (2008).
153. Zhang, W., Wen, H., Li, J., Lin, R. & McManus, D. P. Immunology and Immunodiagnosis of Cystic Echinococcosis: An Update. Clin. Dev. Immunol. **2012,** 1–10 (2012).
154. Kazuko, S., Fasheng, L., Ryan, L. & Steven, S. Fed and Fasted Gastric pH and Gastric Residence Time in Conscious Beagle Dogs. J. Pharm. Sci. **99,** 4215–4227 (2008).
155. Zhang, W., Ross, A. G. & McManus, D. P. Mechanisms of immunity in hydatid disease: implications for vaccine development. J. Immunol. **181,** 6679–6685 (2008).
156. Siracusano, A., Delunardo, F., Teggi, A. & Ortona, E. Host-Parasite Relationship in Cystic Echinococcosis: An Evolving Story. Clin. Dev. Immunol. **2012,** 1–12 (2012).
157. Sjöberg, a. P., Trouw, L. a. & Blom, a. M. Complement activation and inhibition: a delicate balance. Trends Immunol. **30,** 83–90 (2009).
158. Díaz, A., Casaravilla, C., Allen, J. E., Sim, R. B. & Ferreira, A. M. Understanding the laminated layer of larval *Echinococcus* II: immunology. Trends Parasitol. **27,** 264–273 (2011).
159. Peng, X. et al. Detection of Osteopontin in the pericyst of human hepatic *Echinococcus granulosus*. Acta Trop. **100,** 163–171 (2006).
160. Ramos, a. L., Discipio, R. G. & Ferreira, a. M. Eosinophil cationic protein damages protoscoleces in vitro and is present in the hydatid cyst. Parasite Immunol. **28,** 347–355 (2006).
161. Hernández, a, O'Connor, J. E. & Mir, a. Phenotypic analysis of peripheral lymphocyte subpopulations in hydatid patients. Parasitol. Res. **85,** 948–50 (1999).
162. Noël, W., Raes, G., Ghassabeh, G. H., De Baetselier, P. & Beschin, A. Alternatively activated macrophages during parasite infections. Trends Parasitol. **20,** 126–133 (2004).
163. Vuitton, D. a. The ambiguous role of immunity in echinococcosis: Protection of the host or of the parasite? Acta Trop. **85,** 119–132 (2003).
164. Moore, K. W., Malefyt, R. D. W., Robert, L. & Garra, A. O. Interleukin -10 and the interleukin -10. Mol. Cell. Biol. **1,** 683–765 (2001).
165. Period, L. Latent Period of 53 Years in a Case of Hydatid Cyst Disease Hydatid. (2015).
166. Rigano, R. et al. *Echinococcus granulosus* Antigen B Impairs Human Dendritic Cell Differentiation and Polarizes Immature Dendritic Cell Maturation towards a Th2 Cell

Response. Infect. Immun. **75,** 1667–1678 (2007).
167. Mantovani, A. et al. Cancer-related inflammation. Nature **454,** 436–44 (2008).
168. Martinez, F. O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression. J. Immunol. **177,** 7303–7311 (2006).
169. Dematteis, S., Baz, A. & Nieto, A. Antibody and Th1 / Th2-type responses in BALB / c mice inoculated with live or dead *Echinococcus granulosus* protoscoleces. 19–26 (1999).
170. Baz, A., Richieri, A., Puglia, A., Nieto, A. & Dematteis, S. Antibody response in CD4-depleted mice after immunization or during early infection with *Echinococcus granulosus*. Parasite Immunol **21,** 141–150 (1999).
171. Esmaelizad, M. et al. Induction of protective T-helper 1 immune responses against *Echinococcus granulosus* in mice by a multi-T-cell epitope antigen based on five proteins. Mem. Inst. Oswaldo Cruz **108,** 408–13 (2013).
172. Rossi, A. & Marquésa, J. *Echinococcus granulosus* : different cytokine profiles are induced by single versus multiple experimental infections in dogs. Exp. … **130,** 110–115 (2011).
173. Torgerson, P. R. Mathematical models for the control of cystic echinococcosis. Parasitol. Int. **55,** S253–S258 (2006).
174. Fraize, M. et al. Immunogenicity of two *Echinococcus granulosus* antigens EgA31 and EgTrp in mice. Parasitol. Res. **96,** 113–120 (2005).
175. Esteves, A., Señorale, M. & Ehrlich, R. A tropomyosin gene is differentially expressed in the larval stage of *Echinococcus granulosus*. Parasitol. Res. **89,** 501–502 (2003).
176. Stamm, S. et al. Function of alternative splicing. Gene **344,** 1–20 (2005).
177. Alvite, G. & Esteves, A. *Echinococcus granulosus* tropomyosin isoforms: From gene structure to expression analysis. Gene **433,** 40–49 (2009).
178. Martin, R. M., Csar, X. F., Gasser, R. B., Felleisen, R. & Lightowlers, M. W. Myophilin of *Echinococcus granulosus*: isoforms and phosphorylation by protein kinase C. Parasitology **115 (Pt 2,** 205–211 (1997).
179. Soisson, L. M. et al. Induction of protective immunity in mice using a 62-kDa recombinant fragment of a Schistosoma mansoni surface antigen. J Immunol **149,** 3612–3620 (1992).
180. Yang, W., Zheng, Y. Z. Z., Jones, M. K. K. & Mcmanus, D. P. P. Molecular characterization of a calponin-like protein from Schistosoma japonicum. Mol. Biochem. … **98,** 225–237 (1999).
181. Mühlschlegel, F., Liljane, S., Petra, F., Paola, M. & Matthias, F. Paramyosin of *Echinococcus granulosus*: cDNA sequence and characterization of a tegumental antigen. (1993).
182. Fu, Y. et al. A new potent antigen from *Echinococcus granulosus* associated with muscles and tegument. Mol. Biochem. Parasitol. **102,** 43–52 (1999).
183. Vlamakis, H., Aguilar, C., Losick, R. & Kolter, R. Control of cell fate by the formation of an architecturally complex bacterial community. Genes Dev. **22,** 945–953 (2008).
184. Guérout-Fleury, A. M., Frandsen, N. & Stragier, P. Plasmids for ectopic integration in Bacillus subtilis. Gene **180,** 57–61 (1996).
185. Saboulard, D., Lahmar, S., Petavy, A. F. & Bosquet, G. The *Echinococcus granulosus* antigen EgA31: Localization during development and immunogenic properties. Parasite Immunol. **25,** 489–501 (2003).
186. Yasbin, R. E. & Young, F. E. Transduction in *Bacillus subtilis* by bacteriophage SPP1. J. Virol. **14,** 1343–1348 (1974).
187. Cutting, S. M. & P.B., V. H. Genetic analysis. (1990).
188. Nicholson, W. L. & Setlow, P. Molecular biolocigal methods for Bacillus. (1990).
189. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. **76,** 4350–4 (1979).

190. Renart, J., Reiser, J. & Stark, G. R. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc. Natl. Acad. Sci. U. S. A. **76,** 3116–3120 (1979).
191. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685 (1970).
192. Wild, P., Schraner, E. M., H, A. & BM, H. Enhanced resolution of membranes in cultured cells by cryoimmobilization and freeze-substitution. Microsc. Res. Tech. **53,** 313–321 (2001).
193. Schneider, C. a, Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods **9,** 671–675 (2012).
194. Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. The ImageJ ecosystem: An open platform for biomedical image analysis. Mol. Reprod. Dev. **in press,** DOI: 10.1002/mrd.22489 (2015).
195. Saalfeld, S., Pietzsch, T., Preibisch, S. & Tomanc, P. ImgLib2 — generic image processing in Java ˇa. **28,** 3009–3011 (2012).
196. Johannes Schindelin et al. Fiji: an open-source platform for biological-image analysis. Nature **9,** 187 (2012).
197. Shan, M. et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science **342,** 447–53 (2013).
198. Close, B. et al. Recommendations for euthanasia of experimental animals: Part 2. DGXT of the European Commission. Lab. Anim. **31,** 1–32 (1997).
199. Vogt, C. M., Schraner, E. M., Aguilar, C. & Eichwald, C. Heterologous expression of antigenic peptides in *Bacillus subtilis* biofilms. Microb. Cell Fact. 1–12 (2016). doi:10.1186/s12934-016-0532-5
200. Earl, A. M., Losick, R. & Kolter, R. Ecology and genomics of *Bacillus subtilis*. Trends Microbiol. **16,** 269–275 (2008).
201. Härtl, B., Wehrl, W., Wiegert, T., Homuth, G. & Schumann, W. Development of a New Integration Site within the *Bacillus subtilis* Chromosome and Construction of Compatible Expression Cassettes. Society **183,** 2696–2699 (2001).
202. Shan, M. et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science **342,** 447–53 (2013).
203. Torgerson, P. R. Canid immunity to Echinococcus spp.: Impact on transmission. Parasite Immunol. **28,** 295–303 (2006).
204. Smith, J., Lipsitch, M. & Almond, J. W. Vaccine production, distribution, access and uptake. Lancet **18,** 1199–1216 (2011).
205. Zhang, W. et al. Vaccination of dogs against *Echinococcus granulosus*, the cause of cystic hydatid disease in humans. J. Infect. Dis. **194,** 966–974 (2006).
206. Eckert, J., Conraths, F. J. & Tackmann, K. Echinococcosis: An emerging or re-emerging zoonosis? Int. J. Parasitol. **30,** 1283–1294 (2000).
207. P. Economidesa G. Christofia, M.A. Gemmellb, *. Control of *Echinococcus granulosus* in Cyprus and\rcomparison with other island models. Vet. Parasitol. **79,** 151–163 (1998).
208. Kolotilin, I. et al. Plant-based solutions for veterinary immunotherapeutics and prophylactics. Vet. Res. **45,** 117 (2014).
209. Gombold, J. et al. Lot Release and Characterization Testing of Live-Virus–Based Vaccines and Gene Therapy Products, Part 1. Bioprocess Int. APRIL**,** 46–66 (2006).
210. Gemmell, M. a. Natural and acquired immunity factors interfering with development during the rapid growth phase of *Echinococcus granulosus* in dogs. Immunology **5,** 496–503 (1962).
211. Herd, R. P., Chappel, R. J. & Biddell, D. Immunization of dogs against *Echinococcus granulosus* using worm secretory antigens. Int. J. Parasitol. **5,** 395–399 (1975).
212. Herd, R. P. Resistance of dogs to *Echinococcus granulosus*. Int. J. Parasitol. **7,** 135–138 (1977).
213. Woollard, D. J., Gauci, C. G., Heath, D. D. & Lightowlers, M. W. Protection against

hydatid disease induced with the EG95 vaccine is associated with conformational epitopes. Vaccine **19,** 498–507 (2000).
214. Flanigan, T. P., King, C. H., Left, R. R., Nanduri, J. & Mahmoud, a. a F. Induction of resistance to Schistosoma mansoni infection in mice by purified parasite paramyosin. J. Clin. Invest. **83,** 1010–1014 (1989).
215. Nanduri, J. & Kazura, J. W. Clinical and laboratory aspects of filariasis. Clin. Microbiol. Rev. **2,** 39–50 (1989).
216. Li, B.-W., Chandrashekar, R. & J. Weil, G. Vaccination with Recombinant Filarial Paramyosin Induces Partial Immunity to Bvugia malayi Infection in Jirds. J. Immunol. **150,** 1881–1885 (1993).
217. MARK J TAYLOR, R. E. J. A. E. B. Protective immunity induced by vaccination with Onchocerca volvulus tropomyosin in rodents. Parasite Immunol. **18,** 219–225 (1996).
218. Andre, F. et al. Vaccination greatly reduces disease, disability, death and inequity worldwide. Bull. World Health Organ. **86,** 140–146 (2008).
219. Fraser, a, Goldberg, E., Acosta, C. J., Paul, M. & Leibovici, L. Vaccines for preventing typhoid fever. Cochrane Database Syst. Rev. CD001261 (2007). doi:10.1002/14651858.CD001261.pub2
220. Duc, L. H. & Cutting, S. M. Bacterial spores as heat stable vaccine vehicles. Expert Opin. Biol. Ther. **3,** 1263–1270 (2003).
221. Nguyen, A. T. Van et al. *Bacillus subtilis* Spores Expressing the VP28 Antigen: A Potential Oral Treatment to Protect Litopenaeus vannamei Against White Spot Syndrome. FEMS Microbiol. Lett. **358,** 202–208 (2014).
222. Batista, M. T. et al. Gut adhesive *Bacillus subtilis* spores as a platform for mucosal delivery of antigens. Infect. Immun. **82,** 1414–1423 (2014).
223. Cutting, S. M., Hong, H. A., Baccigalupi, L. & Ricca, E. Oral vaccine delivery by recombinant spore probiotics. Int. Rev. Immunol. **28,** 487–505 (2009).
224. Wheelock, A. et al. Socio-psychological factors driving adult vaccination: a qualitative study. PLoS One **9,** e113503 (2014).
225. Chen, D. & Kristensen, D. Opportunities and challenges of developing thermostable vaccines. Expert Rev. Vaccines **8,** 547–557 (2009).
226. Kristensen, D. Summary of stability data for licensed vaccines. Path 17 (2012). at <http://www.path.org/publications/files/TS_vaccine_stability_table.pdf>
227. Amuguni, J. H. et al. Sublingually administered *Bacillus subtilis* cells expressing tetanus toxin C fragment induce protective systemic and mucosal antibodies against tetanus toxin in mice. Vaccine **29,** 4778–4784 (2011).
228. Lee, S. et al. Efficacy, heat stability and safety of intranasally administered *Bacillus subtilis* spore or vegetative cell vaccines expressing tetanus toxin fragment C. Vaccine **28,** 6658–6665 (2010).
229. Serrano, M., Zilhão, R. & Ricca, E. A *Bacillus subtilis* Secreted Protein with a Role in Endospore Coat Assembly and Function
. **181,** 3632–3643 (1999).
230. Newman, J. A., Rodrigues, C. & Lewis, R. J. Molecular Basis of the Activity of SinR Protein, the Master Regulator of Biofilm Formation in *Bacillus subtilis*. J. Biol. Chem. **288,** 10766–10778 (2013).
231. Johansson-Lindbom, B. & Agace, W. W. Generation of gut-homing T cells and their localization to the small intestinal mucosa. Immunol Rev **215,** 226–242 (2007).
232. Flehmig, B. et al. Early appearance of neutralizing antibodies after vaccination with an inactivated hepatitis A vaccine. J. Infect. **35,** 37–40 (1997).
233. Thompson, J. S. Simplification of the Hepatitis B Vaccination Schedule. Am. J. Gastroenterol. **96,** 2001 (2001).
234. Wherry, E. J., Puorro, K. A., Porgador, A. & Eisenlohr, L. C. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. J. Immunol. **163,** 3735–45 (1999).

235. Bikash, S. et al. Activation of B Cells by a Dendritic Cell-Targeted Oral Vaccine. Curr. Pharmacolical Biotechnol. **18,** 1199–1216 (2013).
236. Atarashi, K. et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. Science (80-.). **331,** 337–342 (2011).
237. Smith, P. M. et al. The microbial metabolites, short chain fatty acids, regulate colonic Treg cell homeostasis. Science (80-.). **341,** (2013).
238. Lathrop, S. K. et al. Peripheral education of the immnue system by colonic commensal microbiota. Nature **478,** 250–254 (2011).
239. Gottschalk, R. A., Corse, E. & Allison, J. P. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. J. Exp. Med. **207,** 1701–1711 (2010).
240. Turner, M. S., Kane, L. P. & Morel, P. a. Dominant role of antigen dose in CD4+ Foxp3+ regulatory T cell induction and expansion. J. Immunol. **183,** 4895–4903 (2009).
241. Joffre, O. P., Segura, E., Savina, A. & Amigorena, S. Cross-presentation by dendritic cells. Nat. Rev. Immunol. **12,** 557–569 (2012).
242. Alloatti, A., Kotsias, F., Magalhaes, J. G. & Amigorena, S. Dendritic cell maturation and cross-presentation: timing matters! Immunol. Rev. **272,** 97–108 (2016).
243. Štefanić, S. et al. Polymerase chain reaction for detection of patent infections of *Echinococcus granulosus* ("sheep strain") in naturally infected dogs. Parasitol. Res. **92,** 347–351 (2004).
244. Gottstein, B. & BAFU. Einstufung von Organismen Modul 3: Parasiten. Einstufung von Org. 1–203 (2013).
245. Deplazes, P., Penhale, W. J., Greene, W. K. & Thompson, R. C. a. Effect on humoral tolerance (IgG and IgE) in dogs by oral administration of ovalbumin and Der p I. Vet. Immunol. Immunopathol. **45,** 361–367 (1995).
246. Luzina, I. G. et al. Regulation of inflammation by interleukin-4: a review of "alternatives." J. Leukoc. Biol. **92,** 753–764 (2012).
247. Marchi, L. F. Interferon-gamma enhances phagocytosis, the production of reactive oxygen species and pro-inflammatory cytokines – implications for innate and acquired immunity. Inflamm. Cell Signal. **1,** 10–14800/ics.113 (2014).
248. Zhang, F., Huang, G., Hu, B., Song, Y. & Shi, Y. Induction of immune tolerance in asthmatic mice by vaccination with DNA encoding an allergen-cytotoxic T lymphocyte-associated antigen 4 combination. Clin. Vaccine Immunol. **18,** 807–814 (2011).
249. Kamradt, T. & Mitchison, N. a. Tolerance and autoimmunity. N. Engl. J. Med. **344,** 655–664 (2001).
250. Aeberli, D. et al. Inhibition of the TNF-pathway: use of infliximab and etanercept as remission-inducing agents in cases of therapy-resistant chronic inflammatory disorders. Swiss Med. Wkly. **132,** 414–22 (2002).

ANNEX I: Mouse Animal Experiment Permission

Kopie

Veterinäramt

gesundheitsdirektion
kanton zürich

Obstgartenstrasse 21
CH-8090 Zürich
Tel.: 043 259 41 41
Fax: 043 259 41 40
E-mail: kanzlei@veta.zh.ch

Unser Zeichen: Law
Rückfragen an: Dr. Claudia Lawnitzak
E-mail: claudia.lawnitzak@veta.zh.ch
Vertretung:
Nr: 2012104

A-Post

Frau
Catherine Eichwald
Universität Zürich
Institut für Virologie
Winterthurerstr. 266a
8057 Zürich

Tierschutz/Tierversuche	Form. **B**
Bewilligung / Entscheid der Behörde	Art. 18 Eidg. Tierschutzgesetz; TSchG; Art. 139-141 Eidg. Tierschutzverordnung, TSchV
104/2012 (Int.4787)	Ersetzt Bewilligung vom 20.06.2012.
Bewilligungsinhaber/-in	C. Eichwald
Versuchsleiter/-in	Prof. Dr. M. Ackermann
Titel des Versuchs	**Use of recombinant Bacillus subtilis spores, as a safe carrier, for enteric immunization against Echinococcus granulosus in mice**
Datum der Verfügung	**26.06.2012**
Ablauf der Gültigkeit	**20.07.2015**

Entscheid, Bedingungen und Auflagen

21 Über das beiliegende Gesuch wird wie folgt entschieden:

☒ **Bewilligt mit Auflagen**

22 Folgende **Bedingungen** und **Auflagen** bilden Bestandteil der Bewilligung / des Entscheids:

☒ Personelle Voraussetzungen

- Es gilt die Gesuchsversion 3 vom 30. April 2012.
- Die Angaben im Schreiben (email) vom 30. April 2012 und 18. Juni 2012 bilden integralen Bestandteil der Bewilligung.
- Sollen weitere Personen unter dieser Bewilligung Tierversuche durchführen, so ist dies unter Angabe der Personalien, für neu zu meldende Personen mit einem kurzen curriculum (Fachkenntnisse und bisherige tierexperimentelle Tätigkeiten) sowie den notwendigen Aus- und Weiterbildungsnachweisen dem Veterinäramt vor Aufnahme der Arbeit schriftlich zu melden. Weitere Auflagen betreffend neuem Personal bleiben vorbehalten (Standardauflage gemäss Richtlinie).

- Der Versuchsleiter bzw. die Versuchsleiterin ist namentlich verantwortlich, dass ausschliesslich Personen Tierversuche durchführen, die die Anforderungen an die Aus- und Weiterbildung nach der Tierschutzverordnung sowie den Richtlinien ‚Fachpersonal Tierversuche' (BVET 800.116-1.09, 4. 11.2004) erfüllen (Standardauflage gemäss Richtlinie).

23 **Abweichungen** von den Haltungsvorschriften (Art, Dauer), den Vorschriften über die Herkunft der Tiere und den Vorschriften betreffend die Ausbildung des Fachpersonals werden wie folgt bewilligt:

Berichte und Versuchsänderungen

Art. 145 Abs. 2, 3 TSchV

31 Der/die verantwortliche Bereichsleiter/in hat den Abschluss sowohl des bewilligungspflichtigen wie des nichtbewilligungspflichtigen Versuchs mit dem **Formular C** innert zwei Monaten zu melden. Bei mehrjährigen Versuchen hat die Mitteilung mit den entsprechenden Angaben für das abgelaufene Kalenderjahr bis spätestens Ende Februar zu erfolgen.

32 Bevor Abweichungen vom Versuch gemäss Angaben in den Ziffern 31 – 58 des Gesuchs und den Ziffern 21 - 23 dieser Bewilligung / dieses Entscheids vorgenommen werden (wie Änderung der Methode und Versuchsdauer, Einbeziehen anderer Tierarten, Erhöhung der Tierzahlen, Wechsel Bewilligungsinhaber/-in oder Versuchsleiter/-in), ist beim Veterinäramt eine Ergänzungsbewilligung einzuholen.

Gebühren

Fr.	295.00	Grundgebühr (bis 1 Stunde Aufwand, Laufzeit 1 Jahr, belastender Versuch)
Fr.	120.00	Gebühr pro zusätzlichem Jahr Laufzeit (belastender Versuch): 60.-
Fr.	73.50	Bearbeitungsgebühr für zusätzlichen Aufwand pro Stunde: 147.-
Fr.	126.00	Bewirtschaftungsgebühr für die laufende Bewilligung
Fr.	32.00	Schreibgebühr
Fr.	646.50	Total

Zuwiderhandlungen / Rechtsmittel

Art. 28 Abs. 3 TSchG; Kant. Verwaltungsrechtspflegegesetz

61 Zuwiderhandlungen gegen die Bedingungen und Auflagen werden gemäss Artikel 28 Absatz 3 TSchG bestraft. Die Bestimmung lautet: „Mit Busse wird bestraft, wer durch Unterlassung oder in anderer Weise dem Gesetz, den darauf beruhenden Vorschriften oder einer unter Hinweis auf die Strafandrohung dieses Artikels eröffneten Verfügung vorsätzlich oder fahrlässig zuwiderhandelt".

62 Gegen diese Verfügung kann innert 30 Tagen, von der Mitteilung an gerechnet, bei der Gesundheitsdirektion, Rechtsabteilung, Bereich Rechtsmittel, (Obstgartenstrasse 21, 8090 Zürich) schriftlich Rekurs erhoben werden. Die Rekursschrift muss einen Antrag und dessen Begründung enthalten. Die angefochtene Verfügung ist beizulegen oder genau zu bezeichnen. Die angerufenen Beweismittel sind genau zu bezeichnen und soweit möglich beizulegen.

Kantonales Veterinäramt Zürich

Dr. Claudia Lawnitzak, wiss. Mitarbeiterin

Kopie:
- Bewilligungsinhaber/-in, LTK und Tierschutzbeauftragter
- Bundesamt für Veterinärwesen
- Kant. Tierversuchskommission

Beilage:
- Gesuch, einschliesslich allfällige Zusatzangaben
- Rechnung / interne Verrechnung

Weitere Unterlagen sind im Veterinäramt
☒ vorhanden ☐ nicht vorhanden

Form-A

No. .....
(to be completed by the authorities)

Application for licence to perform animal experiments

Article 18 Animal Welfare Act (TSchG), article 141 Animal Welfare Ordinance (TSchV), article 30 Animal Experimentation Ordinance (TVV)

1 Address of resource manager (institute, company)

Catherine Eichwald
Institute of Virology, Vetsuisse Faculty, University of Zurich
Winthertherurerstrasse 266a, 8057 Zurich, Switzerland
E-mail; tel no. ceichwald@vetvir.uzh.ch; +41 44 635 8711

2 Address of cantonal authority

Kantonales Veterinäramt
Culmanstrasse 1
8090 Zürich

3 **TITLE OF PROJECT**

Use of recombinant Bacillus subtilis spores, as a safe carrier, for enteric immunization against Echinococcus granulosus in mice.

31 Field of study or area of application:

Veterinary Medicine, Bacteriology, Immunology, Parasitology

32
- ☒ *[N]* new application
- ☐ *[F]* application for renewal (no.)
- ☐ *[E]* supplementary application (no.)

In the case of supplementary applications, the requested amendments must be summarized in keywords, the scientific rationale must be indicated in §44.2 and it must be indicated which numbers in the application are affected. The amendments are described alongside the various numbers and must be clearly highlighted versus the existing text:

The modification refers exclusively: ☐ Animal number / species; ☐ Personnel ☐ Validity / extension ☐ Method

33

ANIMAL SPECIES (strain)	**Total number** per application	**Origin*** (a-c)
Balb-c	591	b

*Origin: (a): from previous experiment, please specify licence number:
(b): licensed laboratory animal husbandry in Switzerland (incl. own husbandry): licence number:
or laboratory animal breeder or dealer abroad (art. 118 para. 1, TSchV);
(c): other origin, please specify:

Names and addresses of suppliers:

34.1 **Location of animals:** address, room number
Tierspital Zürich, Nagerzentrum, room B09
Winterthurerstrasse 260,8057 Zurich

licence number of laboratory animal husbandry:
Nr. 147

34.2 **Location of the experiments:** address, room number
Tierspital Zürich, Nagerzentrum room B24
Winterthurerstrasse 260, 8057 Zurich

34.3 **Intercantonal experiment:** yes ☐ no ☒
if so, which other canton(s):

34.4 **Use of genetically modified animals*** : yes ☐ no ☒

*Data sheets for genetically modified lines and strained mutants must be enclosed (additionally for strained lines: decision number).

35 **Maximum prospective degree of severity**: (for details, see para 56.4)

36 **Duration of project:** 3 years

Date of proposed start: Immediately

37 **List of persons who perform or lead measures and procedures in the context of the experiment:**

The annex to this application "Persons involved and certificates of education and training" must be duly filled and copies of education and trainings have to be joined.
Persons who lead or perform experiments must satisfy the educational and further training requirements set forth in art. 132 and 134, para. 1 TSchV and Chapter 3 of the ordinance of the FDEA on qualifications in animal husbandry.

38 The undersigned **resource manager** (art. 129, 130, TSchV) confirms that the persons named in the appendix are familiar with the regulations of the TSchG and TSchV applicable to animal experiments and that they satisfy the educational and further training requirements (art. 130 para. d TSchV).

Place and date	Name and signature
Zurich, January 30th 2012	Dr. Catherine Eichwald

39 **Study director**: if several people perform this function, their areas of responsibility must be defined in the appendix. **Signature of principal study director**: (art. 131, 132 TSchV):

Place and date	Name and signature
Zurich, January 30th 2012	Prof. Dr. Mathias Ackermann

Deputy study director (requirements as stipulated in art. 129 para. 2 TSchV, art. 30e TVV)

Place and date	Name and signature
Zurich, January 30th 2012	Prof. Dr. Peter Deplazes

4 INFORMATION ON THE PURPOSE OF THE EXPERIMENT (for statistics art. 147 TSchV);

sections 41 - 43 indicate **one only in each case** and, if applicable, add a further note or additional information in the case of detailed questions.

41 The project is associated with

- ☒ biological (including medical) studies in the field of basic research
- ☐ discovery, development, and quality control (excluding safety testing) of products or devices for human and veterinary medicine
- ☐ diagnosis of disease
- ☐ education and training
- ☐ protection of humans, animals and the environment by toxicological or other safety tests

  ... for substances used or intended to be used mainly
  - ☐ as pharmaceutical products (including medical devices)
  - ☐ in agriculture
  - ☐ in industry
  - ☐ in private households
  - ☐ as cosmetics or toiletries
  - ☐ as food additives or
  - ☐ to determine potential or actual hazards of contaminants in the general environment, or
  - ☐ other uses. Please specify:

- ☐ other studies. Please specify:

42 The project is associated with

- ☒ human diseases
  - ☐ cancer (excluding carcinogenicity studies)
  - ☐ cardiovascular diseases
  - ☐ nervous and mental disorders
  - ☒ other human diseases. Please specify: Cystic echinococcosis
- ☒ animal diseases. Please specify: Cystic echinococcosis
- ☐ no association with human or animal diseases.

43 The project is associated with procedures required by law (registration and licensing regulations):

- ☐ for Switzerland only
- ☐ for other countries only. Please specify:
- ☐ Both. Please specify:

  Mention the relevant guidelines or test methods:

- ☒ The project has no association with procedures required by law.

44.1 General description of the aim of the project, the status of knowledge and presentation of what is not yet sufficiently known (for example summary of National Science Foundation application, **one page maximum**)

Cystic echinococcosis, also called hydatidosis, represents a worldwide severe public health and livestock problem. The causative agent of the disease is the cestode Echinococcus granulosus, which is known for its two-host life cycle. The adult tapeworm lives in the small intestine of its main host, i.e. carnivores, including dogs. Parasite eggs are shed via feces, a likely mechanism for contamination of plants used for human and/or animal consumption. This may lead to the infection of intermediate host (sheep, cattle, humans). In these animals, larval stages of the metacestode develop and start migrating, reaching internal organs where they persist and may cause the dreaded disease symptoms. To complete the cycle, carnivores are infected following consumption of such persistently infected organs, and the adult tapeworm re-emerges to the ecosystem from the carnivores.

According to mathematical models, the parasite's cycle may be interrupted through successful vaccination of carnivores [Torgerson PR. 2006.Parasite Immunol. 28, 295-303]. An oral vaccination strategy using recombinant Salmonella typhimurium as carrier has previously been developed and tested in dogs. The recombinant bacteria were engineered to produce the E. granulosus antigens EgA31 and EgTrp. Upon immunization, the vaccine conferred only partial protection, i.e. the parasite burden in vaccinated dogs was reduced by 70-80% and the development rate in the remaining worms was also affected [Petavy A-F., et al. 2008. Plos neglected tropical diseases, 2(1):e125]. These data suggest that the antigens used have the desired potential to create an immune response in the host. However, the antigen carrier (Salmonella) has three major drawbacks: (1) the number of live bacteria reaching the duodenum, where immunity is supposed to develop, is dramatically reduced upon passage through the stomach, making the effectiveness of the vaccine unreliable; (2) expression of the recombinant vaccine proteins is unstable because they are encoded on plasmids; (3) there is a general skepticism against Salmonella as vaccine carrier because other members of this genus are associated with disease in humans and animals.

The gram-positive bacteria Bacillus subtilis is used as probiotic in both, humans and animals, and in contrast to Salmonella, it is considered safe for oral use in food supplements [Duc & Cutting. Expert. Opin. Biol. Ther., 2003,3:1263-70; Tam et al., J. Bact. 2006,188:2692-700 and Hong et al. J.Appl. Microbiol, 2008, 160:134-43]. The genetics of B. subtilis sporulation have been extensively studied. In a spore, the chromosome is condensed in the core spore, which is surrounded by layers of lipid membranes and modified peptidoglycan. The spore coat is a very important structure, laminated by a proteinaceous shell that provides resistance against harsh environmental conditions, including heat, acids, organic solvents and enzymes.

TasA, an extracellular protein of B. subtilis, which is secreted but also found in association with both cells and spores, has recently stirred considerable scientific interest. TasA is essential for the formation and structural integrity of B. subtilis biofilms [Aguilar et al. Curr Opin Microbiol. 2007; 10:638-43; Vlamakis et al.Genes Dev, 2008, 22:945-53; Lemon et al.Curr Top Microbiol Immunol. 2008, 322: 1-16 and Romero D et al.,Proc. Natl. Acad. Sci. USA. 2010, 107 (5):2230-4]. This is particularly interesting since there is evidence suggesting that B. subtilis strains could persist in the mouse gut, suggesting that they have adapted to carry out their entire life cycle within the gastrointestinal tract (GIT). This makes TasA an attractive candidate for both antigen presentation and continuous stimulation of immunity. Indeed, there is evidence suggesting that vegetative cells of B. subtilis play a primary role in the development of gut-associated lymphoid tissue (GALT) and somatic diversification of the Ig genes, when these cells were introduced into germ-free appendices of rabbits [Rhee et al. J Immunol. 2004, 172:1118-24]. Subsequent studies demonstrated that orally

administrated B. subtilis spores in mice were able to germinate, proliferate and then re-sporulate in the gut [Tam et al. J Bact. 2006, 188: 2692-700).

B. subtilis is considered a safe probiotic. It may therefore be well accepted in the community; its spores are highly resistant against harsh conditions, which makes them well-suited for their use as oral vaccines; it may form biofilms in the gut, which may be a great advantage for generating a desired local immune response. Finally, a spore-based vaccine would be inexpensive to produce and would not need a cold chain, making it very attractive for commercialization. B. subtilis spores may provide a prototype for generating specific immune responses against various enteric disease organisms.

Mouse model is an excellent and inexpensive tool to detect a humoral response against specific antigens. With this permit, we would like to test in mice a serie of antigens variants (single and combined mixture) from E. granulosus as are: EgTrp, EgA31 and a recently new described candidate as is CRISP(cysteine-rich secreted protein) (Britos et al., Exp. Parasitol. 2007, 116:95-102) and therefore, select the best antigenic variants to be used for dogs oral vaccination (Recombinant B. subtilis: A safe carrier for oral vaccine administration in dogs; permit n° 100/2010). Resulting in a significantive reduction of the number of dogs required for the vaccine trials.

Our experimental design in based in fusioning the E. granulosus antigens (EgTrp, EgA31 and CRISP) to the C-terminal of TasA through molecular biology methodology. The obtained DNA plasmid constructions will be used to transform B. subtilis strain,from which will be obtained the recombinant spores from B. subtilis. These strains will be evaluated for correct expression of the desired antigen in vitro, biofilm formation in vitro and spore production and morphology. All these data must to be analyzed before starting any animal experimentation because is fundamental for certainity of our scientific conclusion.

44.2 Actual question the experiment is designed to answer

Identify through oral immunization of mice, potentially protective antigens of E. granulosus expressed by recombinant B. subtilis. Both qualitative and quantitative aspects of the potentials of individual antigens and antigen combinations will be addressed.

5 **INFORMATION ABOUT THE METHOD** (descriptions and note on sections 51 - 58)

51.1 Overview of the project (experimental design, overview of the method, name of animal model where applicable, the course of the project, flowchart showing sequence of events, biometric planning) (details of the method in para 54)

Experiment #1: Evaluation of an immune response of Balb/C female mice treated with spores expressing peptides from E. granulosus tropomyosin (EgTrp). The aim of this experiment is to determine if mice treated with recombinant B. subtilis spores expressing different peptides of Egtrp are able to induce an immune response against these specific peptides. For this purpose, we will need a total of fourty-eight, six weeks-old Balb-c female mice, divided in 4 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days 1, 21 and 42 with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity 2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores; groups 3 and 4 are mice treated with recombinant B. subtilis spores in whichTasA is fused to peptides (102-107) Egtrp and (102-278) EgTrp, respectively. In this experiment, blood and feces samples will be collected on days 0, 20 , 41 and 50 after the first immunization, to analyse an immune response against the EgTrp protein and to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in feces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

Number of experimental groups	4
Number of mice per group	6
Number of mice per experiment	24
Total number of mice	48

Experiment #2: Evaluation of an immune response of Balb/C female mice treated with spores expressing peptides from E. granulosus paramyosin (EgA31). The aim of this experiment is to determine if mice treated with recombinant B. Subtilis spores expressing different peptides of EgA31 are able to induce an immune response against these specific peptides. For this purpose, we will need a total of sixty,six weeks-old Balb-c female mice, divided in 5 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days: 1, 21 and 42, with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity

2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores; groups 3 , 4 and 5 are mice treated with recombinant B. subtilis spores in whichTasA is fused to peptides (170-369) EgA31, (370-583) EgA31 and (170-585) EgA31, respectively. In this experiment, blood and feces samples will be collected on days 0, 20 , 41 and 50 after the first immunization to analyse an immune response against the EgA31 protein and to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in feces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

Number of experimental groups	5
Number of mice per group	6
Number of mice per experiment	30
Total number of mice	60

Experiment #3: Evaluation of an immune response of Balb/C female mice treated with spores expressing peptides from E. granulosus CRISP. The aim of this experiment is to determine if mice treated with recombinant B. Subtilis spores expressing different peptides of CRISP are able to induce an immune response against these specific peptides. For this purpose, we will need a total of sixty,six weeks-old Balb-c female mice, divided in 5 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days: 1, 21 and 42, with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity 2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores; groups 3 , 4 and 5 are mice treated with recombinant B. subtilis spores in whichTasA is fused to peptides A-CRISP, B-CRISP and C-CRISP, respectively. In this experiment, blood and faeces samples will be taked on days 0, 20 , 41 and 50 after the first immunization to analyse an immune response against the CRISP protein and to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in feces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

Number of experimental groups	5
Number of mice per group	6
Number of mice per experiment	30
Total number of mice	60

Experiment #4: Evaluation of an immune response of Balb/C female mice treated with different ratios of spores expressing TasA fused to E. granulosus peptides tropomyosin (EgTrp) and paramyosin (EgA31). The aim of this experiment is to determine if mice treated with combinations at different ratios of recombinant B. Subtilis spores expressing the best responding TasA fused to EgTrp from experiment #1 and the best responding TasA fused to EgA31 peptides from experiment #2 are able to enhance an immune response against one or two of the specifc antigens in relations to a single spore immunization. For this purpose, we will need a total of eighty-four,six weeks-old Balb-c female mice, divided in 7 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days: 1, 21 and 42, with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity 2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores; group 3, mice treated with recombinant B. subtilis spores with TasA fused with EgTrp (selected from experiment #1);group 4,mice treated with recombinant B. subtilis spores with TasA fused with EgA31 (selected from experiment #2), groups 5, 6 and 7, mice treated with a combination of recombinant B.subtilis spores expressing TasA fused to EgTrp (selected from experiment #1) and recombinant B.subtilis spores expressing TasA fused to EgA31 (selected from experiment #2) at ratios; 1:1 (2.5e10 CFU Egtrp: 2.5e10 CFU EgA31); 2:1 (3.4e10 CFU EgTrp: 1.6e10 CFU EgA31) and 1:2 (1.6e10 CFU EgTrp: 3.4e10 CFU EgA31), respectively. In this experiment, blood and feces samples will be collected on days 0, 20 , 41 and 50 after the first immunization to analyse an immune response against the EgTrp and EgA31 proteins and to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in feces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will

be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

*CFU: colony-formning unit

Number of experimental groups	7
Number of mice per group	6
Number of mice per experiment	42
Total number of mice	84

Experiment #5: Evaluation of an immune response of Balb/C female mice treated with different ratios of spores expressing TasA fused to E. granulosus peptides tropomyosin (EgTrp) and CRISP. The aim of this experiment is to determine if mice treated with combinations at different ratios of recombinant B. subtilis spores expressing the best responding TasA fused to EgTrp from experiment #1 and the best responding TasA fused to CRISP peptides from experiment #3 are able to enhance an immune response against one or two of the specifc antigens in relations to a single spore immunization. For this purpose, we will need a total of eighty-four,six weeks-old Balb-c female mice, divided in 7 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days: 1, 21 and 42, with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity 2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores; group 3, mice treated with recombinant B. subtilis spores with TasA fused with EgTrp (selected from experiment #1);group 4,mice treated with recombinant B. subtilis spores with TasA fused with CRISP(selected from experiment #3), groups 5, 6 and 7, mice treated with a combination of recombinant B.subtilis spores expressing TasA fused to EgTrp (selected from experiment #1) and recombinant B.subtilis spores expressing TasA fused to CRISP (selected from experiment #3) at ratios; 1:1 (2.5e10 CFU Egtrp: 2.5e10 CFU CRISP); 2:1 (3.4e10 CFU EgTrp: 1.6e10CFU CRISP) and 1:2 (1.6e10 CFU EgTrp: 3.4e10 CFU CRISP), respectively. In this experiment, blood and feces samples will be collected on days 0, 20 , 41 and 50 after the first immunization to analyse an immune response against the EgTrp and CRISP proteins and to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in faeces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

Number of experimental groups	7
Number of mice per group	6
Number of mice per experiment	42
Total number of mice	84

Experiment #6: Evaluation of an immune response of Balb/C female mice treated with different ratios of spores expressing TasA fused to E. granulosus peptides paramyosin (EgA31) and CRISP. The aim of this experiment is to determine if mice treated with combinations at different ratios of recombinant B. Subtilis spores expressing the best responding TasA fused to EgA31 from experiment #2 and the best responding TasA fused to CRISP peptides from experiment #3 are able to enhance an immune response against one or two of the specifc antigens in relations to a single spores immunization. For this purpose, we will need a total of eighty-four,six weeks-old Balb-c female mice, divided in 7 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days: 1, 21 and 42, with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity 2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores; group 3, mice treated with recombinant B. subtilis spores with TasA fused with EgA31 (selected from experiment #2);group 4,mice treated with recombinant B. subtilis spores with TasA fused with CRISP (selected from experiment #3), groups 5, 6 and 7, mice treated with a combination of recombinant B.subtilis spores expressing TasA fused to EgA31 (selected from experiment #2) and recombinant B.subtilis spores expressing TasA fused to CRISP (selected from experiment #3) at ratios; 1:1 (2.5e10 CFU EgA31: 2.5e10 CFU CRISP); 2:1 (3.4e10 CFU EgA31:1.6e10 CFU CRISP) and 1:2 (1.6e10CFU EgA31: 3.4e10 CFU CRISP), respectively. In this experiment, blood and faeces samples will be taked on days 0, 20 , 41 and 50 after the first immunization to

analyse an immune response against the EgA31and CRISP proteins and to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in feces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

Number of experimental groups	7
Number of mice per group	6
Number of mice per experiment	42
Total number of mice	84

Experiment #7: Evaluation of an immune response of Balb/C female mice treated with different ratios of spores expressing TasA fused to E. granulosus peptides tropomyosin (EgTrp), paramyosin (EgA31) and CRISP. The aim of this experiment is to determine if mice treated with combinations at different ratios of recombinant B. subtilis spores expressing the best responding TasA fused to EgTrp from experiment #1; recombinant B. subtilis spores expressing the best responding TasA fused to EgA31 from Experiment #2 and the best responding TasA fused to CRISP peptides from experiment #3 are able to enhance an immune response against one, two or three of the specifc antigens in relations to a single or double (experiments #4, #5 and #6) spores immunization. For this purpose, we will need a total of hundred and eight,six weeks-old Balb-c female mice, divided in 9 groups of six mice each. All the groups will be inoculated orally through gavage with an identical volume of a suspension of spores in saline solution (200µl). Animals will be immunized three times at days: 1, 21 and 42, with an amount of 5e10 spores per immunization (Hoang et al., Infection and immunity 2008). The different groups of experimentation will be as follow: group 1, mice treated only with a saline solution; group 2, mice treated with wild-type B.subtilis spores;group 3, mice treated with recombinant B.subtilis spores with TasA fused with EgTrp (selected from experiment #1); group 4, mice treated with recombinant B. subtilis spores with TasA fused with EgA31 (selected from experiment #2);group 5,mice treated with recombinant B. subtilis spores with TasA fused with CRISP (selected from experiment #3), groups 6, 7, 8 and 9, mice treated with a combination of recombinant B.subtilis spores expressing TasA fused to EgTrp(selected from experiment #1); recombinant B.subtilis spores expressing TasA fused to EgA31 (selected from experiment #2) and recombinant B.subtilis spores expressing TasA fused to CRISP (selected from experiment #3) at ratios; 1:1:1 (1.66e10 CFU EgTrp: 1.66e10 CFU EgA31: 1.66e10 CFU CRISP); 1:1:2 (1.25e10 CFU EgTrp: 1.25e10 CFU EgA31: 2.5e10 CFU CRISP); 1:2:1 (1.25e10 CFU EgTrp: 2.5e10 CFU EgA31: 1.25e10 CFU CRISP) and 2:1:1 (2.5e10 CFU EgTrp: 1.25e10 CFU EgA31: 1.25e10 CFU CRISP), respectively. In this experiment, blood and feces samples will be taked on days 0, 20 , 41 and 50 after the first immunization to analyse an immune response against the EgTrp, EgA31 and CRISP proteins, to determine the Ig classes that are produced. Also, the persistence in gut of B.subtilis will be determined by shedding of spores in feces. For this purpose feces will be collected during 7 days after the first immunization. On day 50, mice will be euthanized to analyse the T cell proliferation in spleen and MLN (mesenteric lymph nodes) and histologic analysis of the gut sections. The experiment will be performed twice.

Number of experimental groups	9
Number of mice per group	6
Number of mice per experiment	54
Total number of mice	108

TRAINING ANIMALS: Due that for this kind of immunization strategy is necessary to use gavage needles, that requires certain ability, we ask for 10 female Balb/C mice with the purpose to training students along the duration of the permit period.The students in possession on at least an LTK1E or equivalent certificate, will be trained by Catherine Eichwald. Note: Only people trained in oral administration (as gavage) will be allowed to immunize animals.

Total number of mice	10

SETTINGS FOR T CELL PROLIFERATION ASSAY: This assay, that is required in the experiments #1 and to #7, needs to be set in the lab. Two aspects are essential to be determined for this assay: 1) isolation of T cells from spleen and MLN (required amount of animals: 10 mice, Balb-c female from 6 weeks to 6 months old) (Kruisbeek AM. Curr. Prot. Immunol. (2000):3.1.1-3.1.5) and 2)T cell proliferation conditions assay settings. As described in literature (Kruisbeek AM et al., Curr. Prot. Immunol. (2004), 3.12.1-3.12.20), the culture period required for the stimulation (after cells labeling) varies for different laboratories, media and types

of responding and stimulator cells. Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell proliferation. For this purpose, it will be necessary: 15 mice Balb-c females from 6 weeks to 6 months old.

Total number of mice: 25

ACCESORY SPLEEN CELLS FOR MLN PROLIFERATION ASSAY: Purified T cells, as those purified from MLN, require an exogenous source of accessory non-T cells (Kruisbeek AM et al., Curr. Prot. Immunol. (2004), 3.12.1-3.12.20). Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals". They are not required for cell preparations primed against cellular antigens, because accessory cell functions is provided by stimulator cells. For this purpose, It is required two mice (Balb-c, females, 6 weeks to 6 months old) per experiment. Each experiment will be performed twice.

number of mice per experiment: 4

number of experiments: 7

Total number of mice: 28

For the scheme of the experimental planning, please see the appendix II section

51.2 Reason for selection of method or model, showing its peculiarities / advantages (art. 137 para. 3 TSchV)

The methodology used is the standard for immunization procedure.

51.3 Reason for selecting animal species and, where applicable, for using animals not bred for experimental purposes

Mouse strain: Balb/c. This mouse strain is normally used for immunization trials.

52 Preparation of animals for the experiment (initial examination art. 135 para. 3 TSchV; adaptation art. 119 para. 1 TSchV; type of tagging art. 120 TSchV and art. 5 para. 2 TVV (incl. justification for invasive methods), conditioning, feed or water withdrawal, pre-treatment etc.)

The mice will be kept at the rodent center of the Vetsuisse faculty. During the experimentation procedure the collection of feces is required, for this purpose each mouse will be in an individual cage. Animals will be individualized by ear notching. The animals will be six weeks old at the initiation of each experiment and they will have one week of adaptation before the inititiation of the experiment. Mice will have ad libitum access to sterile food and water.

53.1 Anaesthesia and/or other means of controlling pain (preparations, doses, route and frequency of administration, period of time etc.) (art. 135 para. 5 TSchV)

No anesthesia is required during the whole experimentation procedure. At exception of the ending of each experiment as part of the euthanasia procedure, in which animals will be inconcious previous the cervical dislocation.

The animals will be immunized through gavage, meaning that a precise dose of spore need to be administrated to each rodent into the gastric cavity. For this purpose a gastric feeding needle with a ball tip will be used. This helps to prevent the introduction of the needle into the trachea, preventing further trauma to the oral cavity. No anestesia is required for this procedure(Hedrich, HJ and Bullock GR. The Laboratory mouse. Academic Press, 2004). Additionally, blood sample collection will be performed by tail vein incision. This methodology allowed the collection of 200µl of blood once a week. Pressure will be applied after blood collection to prevent hematomas.

53.2 Reason for selection of anaesthesia and/or analgesia or, if applicable, reason not to use such measures

For euthanasia procedure (cervical dislocation), the anesthetic used is isoflurane with a vaporizator (induction: 5% isoflurane and 4% oxygen; mantention: 2% isoflurane and 3% oxygen)

54.1 Type of procedures/manipulations and parameters to be measured in the animal (indicate sequence of events for individual animal/for animal group): surgical procedures (sequence), substance administration (method and site, amount and frequency), infections, physical treatments (radiation, etc.), follow-up examinations, sampling, reaction tests etc. Details may be provided by means of Standard Operating Procedure (SOP))

In all the experiment (#1 to #7), same kind of procedure will be used, corresponding to:

1) Oral immunization through gavage needle using a maximum volume of 200µl of recombinant B. subtilis spores in saline solution. Immunization will be performed 3 times on days 1, 21 and 42.

2)Blood sample collection will be performed by tail vein incision. This methodology allowed the collection of 200µl of blood once a week. Pressure will be applied after blood collection to prevent hematomas. Blood samples will be collected 4 times during each experiment, on days 0, 20, 41 and 50 on the experimentation schedule.

3)Feces collection are required to mesure secretory antibodies into the feces of the animals and also, to measure the persistence of B.subtilis in the gut of the animals. In both cases, the mice need to be isolated into individual cages (16 cmx 21.5 cm SPF cages) in which, they will isolated for 1 day previous each scheduled immunization (days: 0, 20, 41 and 50) andfor 7 days after the first immunization (days: 1, 2, 3, 4, 5, 6 and 7), respectively. Animals will be regrouped immediately in its original experimental groups (maximun number of mice per cage : 6 ; size SPF cage 19 cm x 37 cm). Please note: Never will be used metabolic cages in these experiments.

54.2 Duration of the series of experiments: total duration of experiment / experiment series (tabulated in an appropriate form where useful): full period of experiment for each individual group or animal, incl. period during which the animal is exposed to substances or other noxae. If animals are to be used repeatedly, indicate the interval between experiments

Time schedule for experiments #1 to #7:

Mice will be immunized on days 1, 21 and 42 by gavage oral administration with 200µl of a saline solution containing 5e10 CFU of recombinant spores.

Mice blood samples will be performed on days 0 (pre-immune), 20, 41 and 50 through tail venipuncture.

Mice feces samples, for antibody detection, will be acquired on days 0, 20, 41 and 50. To allowed the feces collection mice will be isolated during 24 hours into individual cages (NOTE: NO METABOLIC CAGES ARE REQUIRED FOR THIS PURPOSE) and then, immediately regrouped with its respective mates.

For gut persistance of B.subtilis, feces collection is required for individual mouse. This will be accomplished on days 1, 2, 3, 4, 5, 6 and 7 of the immunization schedule (NOTE: NO METABOLIC CAGES ARE REQUIRED FOR THIS PURPOSE) and then, immediately regrouped with its respective mates.

On day 50, mice will be euthanasied by cervical dislocation with previous induction to inconciousness with isoflurane.

Therefore, each experiment is comtemplated in a total period of 50 days.

54.3 Number of animals per experiment/series of experiments: number of groups (including all variables, such as doses, periods of time, controls and details on staggering of experiments over time as stipulated in art. 137 para. 4 lit.c TSchV) and numbers of animals per line, per group, sex of animals

Please, see also the section 51.1

Experiment #1: (Mouse strain: Balb/c, females, six weeks old)

Number of experimental groups 4 ; one control group and three spores groups (wt and TasA-EgTrp)

Number of mice per group 6

Number of mice per experiment 24

The experiment will be performed twice

Total number of mice 48

Experiment #2: (Mouse strain: Balb/c,females, six weeks old)

Number of experimental groups 5; one control group and four spores groups (wt and TasA-EgA31)

Number of mice per group 6

Number of mice per experiment 30

The experiment will be performed twice

Total number of mice 60

Experiment #3: (Mouse strain: Balb/c, females, six weeks old)

Number of experimental groups 5; one control group and four spores groups (wt and TasA-CRISP)

Number of mice per group 6

Number of mice per experiment 30

The experiment will be performed twice

Total number of mice 60

Experiment #4: (Mouse strain: Balb/c, females, six weeks old)

Number of experimental groups 7; one control group and six spores groups (wt; TasA-EgTrp and TasA-EgA31)

Number of mice per group 6

Number of mice per experiment 42

The experiment will be performed twice

Total number of mice 84

Experiment #5: (Mouse strain: Balb/c, females, six weeks old)

Number of experimental groups 7; one control group and six spores groups (wt; TasA-EgTrp and TasA-CRISP)

Number of mice per group 6

Number of mice per experiment 42

The experiment will be performed twice

Total number of mice 84

Experiment #6: (Mouse strain: Balb/c, females, six weeks old)

Number of experimental groups 7; one control group and six spores groups (wt; TasA-EgA31 and TasA-CRISP)

Number of mice per group 6

Number of mice per experiment 42

The experiment will be performed twice

Total number of mice 84

Experiment #7: (Mouse strain: Balb/c, females, six weeks old)

Number of experimental groups 9; one control group and eight spores groups (wt; TasA-Egtrp; TasA-EgA31 and TasA-CRISP)

Number of mice per group 6

Number of mice per experiment 54

The experiment will be performed twice

Total number of mice 108

TRAINING ANIMALS:(Mouse strain:Balb/c, females, six weeks old) Please, see note in section 51.1 , training animals.

Total number of mice 10

SETTINGS FOR T CELL PROLIFERATION ASSAY: (Mouse strain: Balb/c, females from 6 weeks to 6 months old)

1) isolation of T cells from spleen and MLN 10

2)T cell proliferation conditions assay settings 15

Total number of mice 25

ACCESORY SPLEEN CELLS FOR MLN PROLIFERATION ASSAY: (Mouse strain: Balb/c, females from 6 weeks to 6 months old).

number of mice per experiment: 4

number of experiments: 7

Total number of mice: 28

TOTAL ANIMALS REQUIRED IN THE PERMIT: 591

54.4 Reason for the planned number of animal per experiment/series of experiments incl. statistical analysis of data (art. 137 para. 4 TSchV)

The experiments were based in the 3R convention reducing the number and groups of experimental animals in a small number possible to be statistically significant. The data will analysed and processed using conventional statistical methods as significance and t-student test.

55 Evaluation of the method (art. 137 para.3 TSchV)

The data obtained (according to score sheet, in appendix I) will be processed and statistically analyzed using conventional statistical methods as significance and t-student test

56.1 Expected effect on the health and well-being of the animals (general condition, activity, water and food intake, pain reactions, duration and course of impairment, further behavioural parameters, growth, expected lethality, etc.)

All the experiments are under a degree of severity I , after the oral administration through gavage of recombinant B. subtilis spores. No effect in the health and well-being of the mice is expected after treatment when compared with not treated animals. Mice wil have ad-libitum amounts intake of water and food.

56.2 Monitoring of well-being of the animals (art. 135 para. 4 TSchV):
- by which person(s)
- frequency
- criteria of evaluation
- documentation (e.g. score sheet as stipulated in art. 144 para. 1 TSchV)
- according to the phase of experiment

The animals will be monitored daily after immunization by Catherine Eichwald and Bogdana Salathe. The criteria of well-being evaluation will be those indicated by the provided score sheet, as body-weight, behavior among other parameters.

56.3 Indications concerning stress-reducing measures and criteria for premature discontinuation of the experiment (criteria for discontinuation; art. 135 para. 1 and 8 TSchV) and criteria for renouncement to reuse animals

B. subtilis spores are already used as probiotic in humans and It is not expected to produce any stress in the animals. However, if for any unknow reason the animals have a lost in body-weight going under 10-15% a euthanasia procedure will be use to discontinue the experiment. The weight measurement will be performed during 3 days after each immunization, due that this is the period in which most of the spores are sheeded. If

there is a weight reduction is observed, the animals will be weighted daily until reach the correct weight or being required the euthanasia.

56.4 Repartition of animals per severity degree (art. 30 j TVV)

Degree of severity 0: for organs obtention from killing of untreated donors.

Degree of severity I: for oral administration of recombinant B.subtilis spores through gavage.

57.1 Indicate the name and/or the number of the husbandry licence
(if there is no licence for keeping laboratory animals indicate: housing and husbandry of animals before, during, between and after individual experiments; space provided, cage type incl. number of animals, structuring of enclosure, run, individual or group housing, feeding and occupation, routine inspections by animal technicians, etc.)

Animals are kept at Nagerzentrum Vetsuisse Faculty Zurich. Husbandary Licence: 147

57.2 Reason for any deviations from conditions in which animals are kept as defined in the animal protection ordinance in the above-mentioned licence for keeping laboratory animals (e.g: feed withdrawal, immobilization, single housing for social animal species)

Mice behavior under isolation is well-tolerated with our experimental conditions. It has not been observed changes in behavior as well as in body-weight during the short period of isolation. In addition, mice are responding well when re-inserted with the other mice of their respective group.

58 Fate of the animals: utilisation of the animals at the end of the (individual) experience; repeated use in the same or another experiment).
Method of euthanasia (substance, doses, route of administration, etc.)

Mice will be euthanised at the end of the experiment by cervical dislocation (with previous inconciousness with isoflurane) and bleeding (Experiments # 1 to #7). At the moment of the euthanasia for the other animals asked if it is more than 10 animals at time, the animals will euthanisied by CO2 inhalation.

6 INFORMATION ON THE REASON FOR THE ANIMAL EXPERIMENT

61 What other experimental methods are known (e.g. from the literature) which would allow corresponding information to be obtained (mention *in vitro* or *in vivo* methods art. 137 para. 2 and 3 TSchV)

Until know there is not an in vitro method able to reproduce the interaction among the gastrointestinal tract and the immune system.

62 Information on whether the project has been / is being appraised and, if so, by which institution/organization

This project is granted by EU grant: Paravac, grant agreement number: 265862/ University number: 75080502 to the Swiss component of the Parasitology and Virology of the Vetsuisse Faculty, University of Zurich.

63 Assessment of the importance of the anticipated information or results in relation to the pain, suffering, injury or anxiety experienced by the animals and injury to the dignity of the animal (art. 3 and 19 para. 4 TSchG,). In this process of weighing up the various interests (art. 26 TVV) the desired benefits as stipulated under sections 44.1 and 44.2 must in particular be assessed and weighed against the stress on the animals as stipulated under sections 56.1 – 56.4

We know from previous studies that, under the proposed experimental conditions, the animals will not experience pain or suffering (Maximum degree of severity I) and will not be affected in their dignity.

In return, we expect to evaluate a novel, potentially principal means of oral immunization in animals, which may dramatically reduce in the future the need of vaccine-related injections in various species.

Moreover, we address this novel approach by working towards a vaccine against E. granulosus, a tape worm of carnivores, which causes disease, suffering, and death in various other species, including humans, which may serve as intermediate hosts for the tape worm's cysts. As of today, no such vaccine is yet available.

During the whole project, mice will have ad libitum food and water. As described above, a series of measures will be performed to prevent pain or, should it unexpectedly arise, shorten the suffering.

Modification in experiments from permit N° 104/2012 (Internal number: 4787): *"Use of recombinant Bacillus subtilis spores, as a safe carrier, for enteric immunization against Echinococcus granulosus in mice".*

With the data obtained during our experimental trials, we concluded that mice were unable to produce a humoral immune response. Revising the related scientific literature and discussing with experts in the area, as PD Dr. A. S. Zinkernagel (Div. of Infection Diseases and Hospital Epidemiology, USZ), it became clear that would be necessary to modify our experimental trial. The main modification will be the inclusion of an oral treatment with broad-spectrum antibiotics, previous to the immunization of the mice with the recombinant spores. The purpose of the antibiotic treatment is to eradicate part of the mouse intestinal microflora in order to give a niche to the orally administrated recombinant spores. Under these new conditions, we will improve the chance of the recombinant spores to germinate and establish in the gut by decreasing the competition with the intestinal microflora. This will hopefully allow for the formation of a biofilm in the gut and for the expression of the antigen of interest required for GALT stimulation. Previous work performed by other research group has included a treatment consisting in an antibiotic cocktail (ampicillin, gentamicin, metronidazole and vancomycin) mixed with the drinking water for 1 to 2 weeks before starting the experiment [Shan M. et al., 2013 and personal communication]. To corroborate a complete eradication of the intestinal microflora faeces will be collected, resuspended to the same concentration and 50 µl will be used for colony forming units assay.

The experiment # 1 to # 7 will keep the same experimental conditions previously described in the permit N°104/2014 but before each immunization, mice will be treated with an antibiotic cocktail, mixed in the drinking water. This antibiotic cocktail will consist in autoclaved water supplemented with the following antibiotics: ampicillin (0.5mg/ml), gentamicin (0.5 mg/ ml), metronidazole(0.5 mg/ml) and vancomycin (0.25 mg/ml), and supplemented with sucralose (4mg/ml) to make it more palatable to the animals. This mix at been used by different research groups and is considered safe, with no health issues detected in mice. At the day of immunization, mice will return to normal drinking water. To further control the well being of the mice during the antibiotic treatment, the body-weight will be measured every three days.

Reference:

M.Shan, M. Gentile, J. R. Yeiser, A. C. Walland, V. U. Bornstein, K. Chen, B. He, L. Cassis, A. Bigas, M. Cols, L. Comerma, B. Huang, J. M. Blander, H. Xiong, L. Mayer, C. Berin, L. H. Augenlicht, A.Velcich, A. Cerutti. **(2013)** Mucus Enhances Gut Homeostasis and Oral Tolerance by Delivering Immunoregulatory Signals. Science 342, 447-453.

ANNEX II: Dog Animal Experiment Permission

Veterinäramt

gesundheitsdirektion
kanton zürich

Obstgartenstrasse 21
CH-8090 Zürich
Tel.: 043 259 41 41
Fax: 043 259 41 40
E-mail: kanzlei@veta.zh.ch

Unser Zeichen: Law
Rückfragen an: Dr. Claudia Lawnitzak
E-mail: claudia.lawnitzak@veta.zh.ch
Vertretung:
Nr: 2010100

A-Post

Herr
Prof. Dr. Mathias Ackermann
Universität Zürich
Institut für Virologie
Winterthurerstrasse 266a
8057 Zürich

Tierschutz/Tierversuche Form. **B**

Bewilligung / Entscheid der Behörde Art. 18 Eidg. Tierschutzgesetz; TSchG;
Art. 139-141 Eidg. Tierschutzverordnung, TSchV

100/2010 (Int.**4332**)

Bewilligungsinhaber/-in Prof. Dr. M. Ackermann

Versuchsleiter/-in Prof. Dr. P. Deplazes

Titel des Versuchs **Recombinant Bacillus subtilis: A safe carrier for oral vaccine administration in dogs**

Datum der Verfügung **04.06.2010**

Ablauf der Gültigkeit **04.07.2011**

Entscheid, Bedingungen und Auflagen

21 Über das beiliegende Gesuch wird wie folgt entschieden:

☒ **Bewilligt mit Auflagen**

22 Folgende **Bedingungen** und **Auflagen** bilden Bestandteil der Bewilligung / des Entscheids:

☒ Personelle Voraussetzungen
☒ Durchführung eines Pilotversuchs

- Die Angaben in den Schreiben vom 6. und 26. Mai 2010 bilden integralen Bestandteil der Bewilligung.
- Sollen weitere Personen unter dieser Bewilligung Tierversuche durchführen, so ist dies unter Angabe der Personalien, für neu zu meldende Personen mit einem kurzen curriculum (Fachkenntnisse und bisherige tierexperimentelle Tätigkeiten) sowie den notwendigen Aus- und Weiterbildungsnachweisen dem Veterinäramt vor Aufnahme der Arbeit schriftlich zu melden. Weitere Auflagen betreffend neuem Personal bleiben vorbehalten (Standardauflage gemäss Richtlinie).
- Der Versuchsleiter bzw. die Versuchsleiterin ist namentlich verantwortlich, dass ausschliesslich Personen Tierversuche durchführen, die die Anforderungen an die

Aus- und Weiterbildung nach der Tierschutzverordnung sowie den Richtlinien ‚Fachpersonal Tierversuche' (BVET 800.116-1.09, 4. 11.2004) erfüllen
(Standardauflage gemäss Richtlinie).

23 **Abweichungen** von den Haltungsvorschriften (Art, Dauer), den Vorschriften über die Herkunft der Tiere und den Vorschriften betreffend die Ausbildung des Fachpersonals werden wie folgt bewilligt:

Berichte und Versuchsänderungen

Art. 145 Abs. 2, 3 TSchV

31 Der/die verantwortliche Bereichsleiter/in hat den Abschluss sowohl des bewilligungspflichtigen wie des nichtbewilligungspflichtigen Versuchs mit dem **Formular C** innert zwei Monaten zu melden. Bei mehrjährigen Versuchen hat die Mitteilung mit den entsprechenden Angaben für das abgelaufene Kalenderjahr bis spätestens Ende Februar zu erfolgen.

32 Bevor Abweichungen vom Versuch gemäss Angaben in den Ziffern 31 – 58 des Gesuchs und den Ziffern 21 - 23 dieser Bewilligung / dieses Entscheids vorgenommen werden (wie Änderung der Methode und Versuchsdauer, Einbeziehen anderer Tierarten, Erhöhung der Tierzahlen, Wechsel Bewilligungsinhaber/-in oder Versuchsleiter/-in), ist beim Veterinäramt eine Ergänzungsbewilligung einzuholen.

Gebühren

Fr.	295.00	Grundgebühr (bis 1 Stunde Aufwand, Laufzeit 1 Jahr, belastender Versuch)
Fr.	126.00	Bewirtschaftungsgebühr für die laufende Bewilligung
Fr.	32.00	Schreibgebühr
Fr.	453.00	Total

Zuwiderhandlungen / Rechtsmittel

Art. 28 Abs. 3 TSchG; Kant. Verwaltungsrechtspflegegesetz

61 Zuwiderhandlungen gegen die Bedingungen und Auflagen werden gemäss Artikel 28 Absatz 3 TSchG bestraft. Die Bestimmung lautet: „Mit Busse wird bestraft, wer durch Unterlassung oder in anderer Weise dem Gesetz, den darauf beruhenden Vorschriften oder einer unter Hinweis auf die Strafandrohung dieses Artikels eröffneten Verfügung vorsätzlich oder fahrlässig zuwiderhandelt".

62 Gegen diesen Entscheid kann innert 30 Tagen von der Mitteilung an gerechnet an die Gesundheitsdirektion des Kantons Zürich rekurriert werden. Der Rekurs ist im Doppel einzureichen und hat einen Antrag sowie dessen Begründung zu enthalten. Der angefochtene Entscheid ist beizulegen.

Kantonales Veterinäramt Zürich

Dr. Claudia Lawnitzak, wiss. Mitarbeiterin

Kopie:
- Bewilligungsinhaber/-in, LTK und Tierschutzbeauftragter
- Bundesamt für Veterinärwesen
- Kant. Tierversuchskommission

Beilage:
- Gesuch, einschliesslich allfällige Zusatzangaben
- Rechnung / interne Verrechnung

Weitere Unterlagen sind im Veterinäramt
☒ vorhanden ☐ nicht vorhanden

Animal experiments Form. **A**

No. .....
(to be completed by the authorities)

Application for licence to perform animal experiments

1 Address of applicant (contact person, institute, firm)

Prof. Dr. Mathias Ackermann
Institute of Virology, Vetsuisse Faculty
Winthertherurerstrasse 266a, 8057 Zürich

E-mail; phone no. mathias.ackermann@vetvir.uzh.ch; 044658702

2 Address of cantonal authority

Katonales Veterinäramt
Culmanstrasse 1, 8090 Zürich

3 **TITLE OF EXPERIMENT / PROJECT**

Recombinant Bacillus subtilis: A safe carrier for oral vaccine administration in dogs

31 Field of study or area of application:

Medicine, veterinary medicine, parasitology, bacteriology, immunology.

32 ☒ *[N]* new application
☐ *[R]* application for renewal (no.)
☐ *[S]* supplementary application (no.) →Amendment ☐ type of method
☐ number of animals / animal species
☐ validity / period of extension
☐ other (**give corresponding figures in application**)

*In the case of supplementary applications, please only note or highlight amendments

33

ANIMAL SPECIES	**Total number** per application	**Source** (a-c)
dog (Beagle)	13	b

Source: (a): from a previous experiment;
(b): from a recognized animal breeding or husbandry facility (article 59b, OPA);
(c): from another source, namely:

Names and addresses of suppliers:

34 **Address of place where animals are kept:** Stigenhof, Embrach
Vetsuisse ZH dog holding facility

Use of genetically modified animals* : yes ☐ no ☒

*Datasheets for registration and characterization of genetically modified animals must be attached.

35 **Maximum expected degree of stress**: 1 (for details: see section 56.4)

36 **Overall duration of project:** 1 year
Scheduled starting date: immediately

37 **List of persons who perform or supervise operations and interventions in the experiment:**

People who supervise or perform experiments must meet the training and continuing education requirements as defined in the "Ordinance on the training and continuing education of specialists for animal experiments" dated 12 October 1998.
The corresponding appendix for "persons involved and certificates of training and continuing education" must be completed and copies of the certificates enclosed.

38 The signing **scientific institute or laboratory director** (article 61a, para. 1, OPA) confirms that the people listed under **section 37** are familiar with the regulations of the LPA and the OPA applicable to animal experiments and that they meet the training and continuing education requirements (article 59d, OPA).

Zürich, 1st March 2009 — Mathias Ackermann

Place and date — Name and signature

39 **Study director**: if more than one person performs this function, their area of responsibility must be defined as described in section 37. **Signature of principal study director**: (article 59d, OPA):

Zürich, 1st March 2010 — Peter Deplazes

Place and date — Name and signature

4 INFORMATION ON THE PURPOSE OR AIM OF THE EXPERIMENT

(for statistics article 64b, OPA); sections 41 – 43, indicate **one only in each case** and, if applicable, add a further note or additional information in the case of detailed questions.

41 The project is associated with

- ☒ biological (including medical) studies in the field of basic research
- ☐ discovery, development, and quality control (excluding safety testing) of products or devices for human and veterinary medicine
- ☐ diagnosis of disease
- ☐ education and training
- ☐ protection of humans, animals and the environment by toxicological or other safety tests

... for substances used or intended to be used mainly

- ☐ as pharmaceutical products (including medical devices)
- ☐ in agriculture
- ☐ in industry
- ☐ in private households
- ☐ as cosmetics or toiletries
- ☐ as food additives or
- ☐ to determine potential or actual hazards of contaminants in the general environment
- ☐ any other use, namely:

- ☐ other studies, namely:

42 The project is associated with

- ☒ human diseases
  - ☐ cancer (excluding carcinogenicity studies)
  - ☐ cardiovascular diseases
  - ☐ nervous and mental disorders
  - ☒ other human diseases, namely: cystic echinococcosis
- ☒ animal diseases, namely: cystic echinococcosis
- ☐ no association with human or animal diseases.

43 The project is associated with procedures required by law (registration and licensing regulations):

- ☐ Switzerland only
- ☐ other countries only, namely:
- ☐ both, namely:

  Mention the relevant guidelines or test methods:

- ☒ The project has no association with procedures required by law.

44.1 Description of the aim of the experiment (for example summary of National Science Foundation study, **maximum one page**):

Cystic echinococcosis, also called hydatidosis, represents a worldwide severe public health and livestock problem. The causative agent of the disease is the cestode Echinococcus granulosus, which is known for its two-host life cycle. The adult tapeworm lives in the small intestine of its main host, i.e. carnivores, including dogs. Parasite eggs are shed via feces, a likely mechanism for contamination of plants used for human and/or animal consumption. This may lead to the infection of intermediate host (sheep, cattle, humans). In these animals, larval stages of the metacestode develop and start migrating, reaching internal organs where they

persist and may cause the dreaded disease symptoms. To complete the cycle, carnivores are infected following consumption of such persistently infected organs, and the adult tapeworm re-emerges to the ecosystem from the carnivores.

According to mathematical models, the parasite's cycle may be interrupted through successful vaccination of carnivores [Torgerson PR. 2006.Parasite Immunol. 28, 295-303]. An oral vaccination strategy using recombinant Salmonella typhimurium as carrier has previously been developed and tested in dogs. The recombinant bacteria were engineered to produce the E. granulosus antigens EgA31 and EgTrp. Upon immunization, the vaccine conferred only partial protection, i.e. the parasite burden in vaccinated dogs was reduced by 70-80% and the development rate in the remaining worms was also affected [Petavy A-F., et al. 2008. Plos neglected tropical diseases, 2(1):e125]. These data suggest that the antigens used have the desired potential to create an immune response in the host. However, the antigen carrier (Salmonella) has three major drawbacks: (1) the number of live bacteria reaching the duodenum, where immunity is supposed to develop, is dramatically reduced upon passage through the stomach, making the effectiveness of the vaccine unreliable; (2) expression of the recombinant vaccine proteins is unstable because they are encoded on plasmids; (3) there is a general skepticism against Salmonella as vaccine carrier because other members of this genus are associated with disease in humans and animals.

The gram-positive bacteria Bacillus subtilis is used as probiotic in both, humans and animals, and in contrast to Salmonella, it is considered safe for oral use in food supplements [Duc & Cutting. Expert. Opin. Biol. Ther., 2003,3:1263-70; Tam et al., J. Bact. 2006,188:2692-700 and Hong et al. J.Appl. Microbiol, 2008, 160:134-43]. The genetics of B. subtilis sporulation have been extensively studied. In a spore, the chromosome is condensed in the core spore, which is surrounded by layers of lipid membranes and modified peptidoglycan. The spore coat is a very important structure, laminated by a proteinaceous shell that provides resistance against harsh environmental conditions, including heat, acids, organic solvents and enzymes. The outermost layer of the spore coat is composed of at least five proteins: CotA, CotB, CotC, CotD and CotG.

Spore coat proteins have already been used as fusion partners to generate successful vaccines against bacterial diseases in murine models, for example: (1) a CotB and CotC fusion to peptides from B. anthracis, and (2) the CotB fusion to the toxoid alpha from Clostridium perfringens [Duc et al.Vaccine.2007, 25:346-55 and Hoang et al.Infect Immunol. 2008, 76:5257-65]. TasA, an extracellular protein of B. subtilis, which is secreted but also found in association with both cells and spores, has recently stirred considerable scientific interest. TasA is essential for the formation and structural integrity of B. subtilis biofilms [Aguilar et al. Curr Opin Microbiol. 2007; 10:638-43; Vlamakis et al.Genes Dev, 2008, 22:945-53; Lemon et al.Curr Top Microbiol Immunol. 2008, 322: 1-16 and Romero D et al.,Proc. Natl. Acad. Sci. USA. 2010, 107 (5):2230-4]. This is particularly interesting since there is evidence suggesting that B. subtilis strains could persist in the mouse gut, suggesting that they have adapted to carry out their entire life cycle within the gastrointestinal tract (GIT). This makes TasA an attractive candidate for both antigen presentation and continuous stimulation of immunity. Indeed, there is evidence suggesting that vegetative cells of B. subtilis play a primary role in the development of gut-associated lymphoid tissue (GALT) and somatic diversification of the Ig genes, when these cells were introduced into germ-free appendices of rabbits [Rhee et al. J Immunol. 2004, 172:1118-24]. Subsequent studies demonstrated that orally administrated B. subtilis spores in mice were able to germinate, proliferate and then re-sporulate in the gut [Tam et al. J Bact. 2006, 188: 2692-700).

B. subtilis is considered a safe probiotic. It may therefore be well accepted in the community; its spores are highly resistant against harsh conditions, which makes them well-suited for their use as oral vaccines; it may form biofilms in the gut, which may be a great advantage for generating a desired local immune response. Finally, a spore-based vaccine would be inexpensive to produce and would not need a cold chain, making it very attractive for commercialization. B. subtilis spores may provide a prototype for generating specific immune responses against various enteric disease organisms. However, there is no information available regarding its behaviour as an oral vaccine-delivery system in dogs. To establish this crucial data, we suggest to perform a series of pilot experiments.

44.2 Basic question which experiment is designed to answer; status of research; presentation if what is not yet sufficiently well-known:

In the present application, we would like to perform a pilot study addressing the following questions: (1) Will recombinant B. subtilis spores safely survive the passage through the stomach of orally vaccinated dogs? (2) Will they germinate in the small intestine and form a biofilm in the gut of those dogs? If yes, which part of the intestine will host the biofilm? (3) Will the dogs raise an immune response against a model recombinant protein (mCherry) delivered through B. subtilis? (4) Will the dogs, orally vaccinated with recombinant B. subtilis, raise an immune response against the E. granulosus antigens EgA31 or EgTrp, when these antigens are genetically fused to TasA?

IMPORTANT NOTICE: The pilot study will be performed in two steps. In the first step, we will address the questions 1, 2, and 3. Based on the knowledge gained there, we will address question 4 in a second step.

5 **INFORMATION ABOUT THE METHOD** (descriptions and note on sections 51 - 59)

51.1 Experimental design (overview of the method, the course of the project, name of animal model where applicable)

EXPERIMENT #1 (outline): To be effective as a vaccine delivery vector, B. subtilis has to overcome the harsh conditions of the passage through the stomach when orally administered. To monitor this first task, we have generated a recombinant B. subtilis that expresses luciferase. Luciferase is a light-emitting protein that can be visualized in vivo by using the IVIS system, a highly sensitive camera that non-invasively and quantitatively detects light even when emitted from within an organism. For the experiment, we will inoculate a small number of dogs with such bacteria before monitoring light emission from within the gut of these animals. Once the bacterial spores have safely passaged through the stomach, they will germinate and start replication. Increasing amounts of light will be emitted with increasing numbers of bacteria. IVIS imaging will allow to monitor over time the anatomical location of highest bacterial replication, which is very important for vaccine efficacy. The second question asks whether or not the bacteria are able to form a biofilm at the desired location. To monitor this event, we have our B. subtilis vector engineered to produce the fluorescent protein mCherry as a fusion protein with TasA (TasA-mCherry). In preliminary in vitro experiments, we have established that this fusion protein is present in high amounts in the biofilm and that, importantly, it does not interfere with biofilm formation. Therefore, we will be able to detect the location and quantify of biofilm formation at the end of the experiment by using fluorescence microscope techniques. Thirdly, we will monitor the sera of the experimental animals for antibody formation against mCherry, which may provide a first hint towards the suitability of our antigen presentation approach in the new system. In addition, collected feces samples will be analyzed for antibodies (potential IgA in feces) against mCherry as well as for the presence of B. subtilis spores, which will be a good indicator for persistence of B. subtilis in the gut.

EXPERIMENT #1 (time table, number of animals, and doses): Animals of up to 700 g bodyweight can be accomodated in the IVIS for monitoring of luciferase activity. Therefore, we will use a group of five, approximately 10 days old Beagle puppies (close to 700g). On days 1, 21 and 42 of the experiment, four animals will be orally immunized with a dose of 5e10 cfu recombinant B. subtilis spores in 1 ml volume of milk, whereas one animal will serve as unimmunized control. Similar doses of Bacillus cereus, a strain of the same genus than Bacillus subtilis, have been reported as having no adverse effects when administrated to dogs as probiotics [Biourge V. et al., J. Nutr. 1998; 128:2730S-2732S]. IVIS monitoring will be done at 2, 6 and 24 hours and 2, 3 and 7 days post-inoculation. The puppies may be narcotized for IVIS monitoring, which will not take longer than 10 minutes per dog and per analysis. Blood for serological analysis will be taken from the jugular vein prior to inoculation as well as once per week until the end of the experiment. For control purposes, blood will also be taken from the bitch. On day 60, the inoculated animals and the negative control will be euthanazied and samples of the gut will be taken for fluorescence microscopy to visualize the biofilms as well as for molecular (PCR detection of B. subtilis) and bacteriological (evaluation of the intestinal flora) analyses as well as histological analyses (potential inflammatory responses, properties of the biofilm, etc.) to determine the state of infection.

EXPERIMENT #2: After careful analysis of the results obtained from the first experiment, we suggest to follow up with a second pilot experiment, this time using B. subtilis expressing a fusion of TasA to the E. granulosus antigens EgA31 or EgTrp. The readout will be very similar to the first experiment (see above) but may require changes in the time tables, age of experimental animals, dose of inoculated spores, etc. Importantly, this second series will provide serological evidence (detection of antibodies against EgA31 or EgTrp or both) as to whether or not our approach might be suitable at all in the context of vaccination against E. granulosus.

EXPERIMENT #2: Since IVIS monitoring is not planned for the second experiment, the age and body weight of the experimental dogs is not as crucial any more. Therefore, the age of the animals can be adjusted to fully guarantee a mature immune system. Two groups of 4 dogs will be immunized three times with a dose of 5e10 cfu of recombinant spores from B. subtilis carrying: a fusion of TasA-EgA31 (group 1) and a fusion of TasA-EgTrp (group 2) in a volume of 1 ml milk solution. A second immunization will be given on day 21, and a third immunization on day 42. Serum samples for detection of antibodies against EgA31 and EgTrp will be taken prior to inoculation and weekly thereafter. Growth and shedding of B. subtilis in the dogs will be monitored by isolation from collected feces. The experiment will be terminated at 60 days after the first inoculation.

For biological safety reasons (Meldung A90029), all feces from immunized dogs that are not used for analyses will be collected and safely eliminated to avoid the uncontrolled release of the spores into the enviroment. At the end of the experiments, the facilities will be desinfected using hypochloride. Wash-off fluid will be collected and incubated with hypochloride (2.6% final concentration) for 15 min before being diluted and discarded.

51.2 Reason for selection of method (or model) showing its peculiarities / advantages

Dogs play a crucial role in the epidemiology of E. granulosus and associated disease of humans and husbandry animals. Thus, successful vaccination of dogs may eliminate or at least reduce the risk of humans and animals to suffer from Cystic Echinococcosis. Oral vaccination has the promise of inducing a robust immune protection, where it is most needed, namely in the gut of dogs, where the tapeworm has established and, thus, where the source of shedding of the parasite's eggs is located. Bacterial spores are known to resist and survive harsh conditions, such as passage through the stomach. Therefore, they are well suited to carry the recombinant antigens to the location in the gut, where immunity should develop. B. subtilis is well accepted as a probiotic and has, therefore, the potential to be commercialized as a vaccine carrier, provided that it proofs to be suitable for oral vaccination purposes. Moreover, spore-vaccines can be produced inexpensively, which makes them also attractive for applications in third world countries.

51.3 Reason for selecting animal species (breeds to be listed) and, where applicable, for using animals not bred for experimental purposes

Dogs are the ideal target for vaccination against E. granulosus. Therefore, the validation of B. subtilis spores as carrier for the specific antigens in dogs is an essential step in the process. The selected breed of dog for this experiment will be the Beagle from our faculity's own breeding facilities. Their puppies at 10 days have a size of approximately 700 g, the limit size requirement for fitting into the IVIS camera chamber.

52 Special preparation of animals for the experiment (adaptation, type of tagging, conditioning, feed or water withdrawal, pretreatment etc.)

The animals will be kept at the dog holding facility of the Vetsuisse faculty, University of Zürich.

The animals will be tagged before weaning through the implantation of a transponder (microchip), as denoted into the Art 59c, TSchV. Additionally, application of the microchips will be carried out by qualified staff.

53.1 Narcosis and/or other analgesics (preparations, doses, route and frequency of administration, period of time etc.)

For experiment #1, narcosis of the puppies is required during the IVIS monitoring procedure

For this purpose, the anesthetic chosed is Sevoflurane at 1.5 MAC (minimum alveolar concentration). This anesthetic is well accepted due to the follow properties: inhalation via mask, colourless, very short acting and only 2-3% of it is metabolized. This procedure will be under the consultation of Prof. Dr. Regula Bettschart DVM, an renowned expert for narcosis in dogs.

53.2 Reason for selection of narcosis or analgesia or, if applicable, reason for not using narcosis or other measures to reduce discomfort (e.g. use of analgesics)

The narcosis will be applied to avoid stressing of the puppies in the IVIS chamber. The IVIS procedure by itself is non-invasive but the puppies need to remain motionless in the dark chamber for adequate picture acquisition. The chamber is equipped for gas application. Each examination will take approximately 10 minutes.

54.1 Type of intervention/manipulation and parameters to be measured in the animal (procedure for individual animal/for animal group): surgical interventions (general procedure), substance administration (method and site, amount and frequency), infections, physical treatments (radiation, etc.), procedural controls, obtaining of samples, reaction tests etc.

Dogs will be immunized orally three times with 5e10 cfu of recombinant spores/dose. This procedure will be performed by application through gavage. The IVIS chamber will be used only after the first immunization.

Time Table Experiment #1

Procedure	Timing	Details
Oral immunization	1, 21,42 (dpi)	5e10 cfu bacterial spores in 1 ml per os
IVIS chamber	2, 6, 24 (hpi) and 2, 3, 7 (dpi)	Narcosis, approximately 10 min per time per dog; prior and post narcosis, the puppies will be kept with their mother
Blood sampling	0, 7, 14, 21, 28, 35, 42, 49, 56, 60 (dpi)	<1 ml/100 g bodyweight

(dpi= day post inoculation; hpi= hour post inoculation)

Time Table Experiment #2

Procedure	Timing	Details
Oral immunization	1, 21,42 (dpi)	5e10 cfu bacterial spores in 1 ml per os
Blood sampling	0, 7, 14, 21, 28, 35, 42, 49, 56, 60 (dpi)	<1 ml/100 g bodyweight

(dpi= day post inoculation)

For more details see also section 51.1.

54.2 Duration of experiment/series of experiments: total duration of experiment for each individual group or animal, incl. period during which the animal is exposed to substances or other noxae.

Experiment #1 and #2 will have a duration of 60 days, each from the first immunization. Making a total of 120 days. This period does not includ the time required for breeding and data analysis.

54.3 Number of animals per experiment/series of experiments: number of groups (including all variables, such as doses, periods of time, controls) and numbers of animals per group

Experiment #1

4 animals to be vaccinated (recombinant spores TasA-mCherry/LuxCDABE)

1 animal as non-vaccinated control

Total animal/experiment: 5

Experiment #2

number of groups: 2 (recombinant spores TasA-EgA31 and recombinant spores Tas-EgTrp)

number animals/group:4

Total animals/experiment: 8

TOTAL ANIMALS FOR WHOLE PROJECT: 13

54.4 Reason for the planned numbers of animals (the breeds of animal used must be indicated)

The numbers of animals used is the minimal number to establish significant terms of the humoral response, IVIS acquired images and histogical analysis from the immunized animals

55 Type of analysis, including biometric planning

The significance of the data obtained will be determined through t-student parameters from the average of the group of samples tested.

56.1 Expected effect on the well-being of the animals (activity, water and food intake, pain reactions, duration and course of harmful impairment, further behavioural parameters, growth, expected lethality, etc.)

During the whole experimental procedure dog bitches and puppies will be provided with ad-libitum water and food. Dogs will be maintained indoors during the whole experimentation in order to meet the following requirements: control of odor and noise, appropriate utilities (as sewage and water); outside windows and skylights; sheltered structures that must be large enough to permit each animal to sit and lie down in a normal manner and to turn around freely; protection from the elements and extreme temperatures at all times. Moreover, in accordance with art. 31, dogs will be allowed to have daily at least 30 minutes of activity in open air. This will include weekends and holidays.

In experiment #1: Puppies will be immunized with 5e10 cfu of the spores/dose once they are 700g (approx. 10 days old). For in vivo imaging (IVIS proceduce), the animals will have to be transported from Stigenhof to the IVIS facility in Zurich. In order to avoid unneccessary stress, the bitch and her entire litter will be transported and kept together with exception of the 10 min required for monitoring, during which time the puppies are under narcosis (sevoflurane, as described in section 53.1). The IVIS facility is equipped to hold animals for short periods. For keeping the animals in between of the first two measurements, quiet and adequate facilities are available. During the whole procedure, the animals will be monitored for glucose status, body temperature, breathing (thorax movement) and pulse.

To further reduce stressing of the bitch, we shall not use entire litters for the experiments (both #1 and #2). At least one or two puppies per bitch will remain excluded from the experiment in order to avoid problems with the bitch's mammary gland and lactation at the end of the expermiment.

Blood sample from puppies will be collected from the jugular vein. Not more than 1ml/100g body weight/week will be taken (due to the immaturity of the sympathic system in neonates, they cannot easily compensate volume loss).

56.2 Monitoring of well-being: frequency, criteria of assessment, documentation (e.g. score sheet) according to phase of experiment

The animal well-being will be followed by a score-sheet as indicated into apendix I

56.3 Criteria for stress-reducing measures and (premature) discontinuation of the experiment (criteria for withdrawal) and for not re-using the animals,

The criteria used to terminate individual animals during the ongoing experiments are described in the score sheet (appendix I). All animals that present three or more of the critical characteristics will immediately be sacrified to avoid unnecessary (though unexpected) suffering.

56.4 Estimated number of animals per severity grade

Severity I: animals immunized with recombinant luminescent spores of Bacillus subtilis and anesthetized to trace and quantify the growth of germinated vegetative bacterial cells in the pup's guts.

Number of animals severity 0: 8

Number of animals severity I: 5

57.1 Husbandry and care of animals before, during, between, and after the various experiments (available space, structure, run, keeping individually or groups, feeding and activity, routine checks by the animal keeper; in the event of repeated use, also indicate the interval between experiments)

The dog bitches and puppies will be kept at the dog holding facility Stigenhof of the Vetsuisse Faculty, University of Zürich. The required conditions for this purpose have been established according to the swiss ordinance. Conventional conditions can be applied, with exception of two hygienic conditions (Meldung A90029): (1) feces is to be collected and to be safely disposed of; (2) decontamination of the facility using hypochloride at the end of the experiment.

The animals will receive sufficient food and water as stipulated by the cf. art2, para 1 TSchV. Additionally, breeding bitches will be housed together with their pups for at least 8 weeks. Young dogs raised in the litter by their mothers can later be kept among congeners. All the dogs before, during and after experiments (this last only for experiment #2) will be housed, fed and supplied with medical attention in keeping with the state article (cf. art 15, para 3 TSchG). The group holding will be kept in the dimension established in appendix 1, Table 152, TSchV.

57.2 Reason for any deviations from conditions in which animals are kept as defined in the animal protection ordinance (incl. feed withdrawal, immobilization)

n/a

58 Method of sacrifice, use of animals after completion the (individual) experiment (repeated use in the same or another experiment)

The animals will be euthanized through a three times overdose of intravenous barbiturate (sodium pentobarbitone) dose (80 mg/kg) [Close, B et al., Recommendatiosn for euthanasia of experimental animals: Part 2. Laboratory Animals (1997) 31, 1-32].

6 INFORMATION ON REASON FOR THE ANIMAL EXPERIMENT

61 What other experimental methods are known (e.g. from the literature) which would allow corresponding information to be obtained (mention any *in vitro* or *in vivo* methods)

There is no in vitro method described in the literature that would allow corresponding information to be obtained.

62 Information on whether the project has been / is being appraised and, if so, by which institution/organization

This project as been applied as a European Community grant as:"…." . Form A for pilot experiments in mice had been approved from cantonal veterinary office with the number: 157/2009.

63 Assessment of the importance of the anticipated information or results obtained in relation to the pain, suffering, injury or anxiety experienced by the animals

This experiment is designed as a pilot study to test a novel potential oral vaccination procedure for dogs. If successful, a whole new generation of oral vaccines for dogs may be envisioned. Thus, in the future, dogs may have to less often encounter the slightly stressful procedure of vaccine injections. Furthermore, this experiment may serve to establish a successful vaccine against E. granulosus.

The experimental dogs will be maintained in high standard care during the whole experimentation period. Pups will be kept all the required time (8 weeks) with the bitch to avoid anxiety and unnecessary stress. As mentioned in section 57.1., young animals from the same litter will be housed with congeners.

Curriculum Vitae

Personal Dates

Last name:	VOGT
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Education

2012 – 2016	Institute of Virology, University of Zurich, Doctoral Student
2010 – 2012	Faculty of Medicine, University of Bern, Master Student
2007 – 2010	Faculty of Science, University of Fribourg, Bachelor Student
2002 – 2006	Kantonsschule Kollegium Schwyz, Schwyz

Diplomas and Degrees

2012	Master of Science (M.Sc.), Biomedical Sciences (Master Thesis: *Development of a Bioartificial Pulmonary Alveolar Membrane*)
2010	Bachelor of Science (B.Sc.), Biomedical Sciences
2006	Matura, Economy and Law

Research Experience

2012 – 2016	Doctoral Student, Institute of Virology, University of Zurich
2011 – 2012	Master Student, ARTORG Center, University of Bern

Publication

Heterologous expression of antigenic peptides in *Bacillus subtilis* biofilms,
Cédric M. Vogt, Elisabeth M. Schraner, Claudio Aguilar, Catherine Eichwald,
Microbial Cell Factories, 2016